THIS PART COMPLETES VOLUME 73 (1959)
DECEMBER 1959, VOLUME 73, No. 4

UNIVERSITY OF MICHIGAN
JAN 14 1960
SCIENCE LIBRARY

THE BIOCHEMICAL JOURNAL

Edited for

THE BIOCHEMICAL SOCIETY

Editorial Board

W. V. THORPE (*Chairman*) H. J. ROGERS (*Deputy Chairman*)

E. P. ABRAHAM C. H. GRAY
W. N. ALDRIDGE D. C. HARRISON
W. BARTLEY J. LASCELLES
K. BURTON L. W. MAPSON
E. A. DAWES R. MARKHAM
R. M. C. DAWSON R. J. PENNINGTON
J. J. SCOTT

Secretary to the Board: F. CLARK

UNIVERSITY OF MICHIGAN
JAN 14 1960
SCIENCE LIBRARY

CAMBRIDGE UNIVERSITY PRESS
BENTLEY HOUSE, 200 EUSTON ROAD, LONDON, N.W. 1
AMERICAN BRANCH: 32 EAST 57TH STREET, NEW YORK 22, N.Y.

Price 25s. net (U.S.A. $4.25)

The *Biochemical Journal* is conducted by the **Biochemical Society** and is published by the Cambridge University Press. The *Journal* is issued in three volumes per year. Each volume consists of approximately 700 pages, published in four parts at intervals of one month. The index for each volume is issued separately.

The Biochemical Society was instituted solely for the advancement of the Science of Biochemistry, and holds meetings at different centres for the communication of original papers, and for the discussion of current problems. Persons interested in Biochemistry are eligible for election. Members whose subscriptions are not in arrears are entitled to receive the *Biochemical Journal* without further payment. Further information may be obtained from the Honorary Secretaries, Dr P. N. Campbell, The Courtauld Institute of Biochemistry, Middlesex Hospital, London, W. 1, and Dr W. J. Whelan, The Lister Institute of Preventive Medicine, Chelsea Bridge Road, London, S.W. 1, or from the Honorary Treasurer, Dr F. A. Robinson, c/o Allen and Hanburys, Ltd., Bethnal Green, London, E. 2.

Subscriptions to the *Biochemical Journal*. For non-members of the Biochemical Society the subscription to the *Biochemical Journal* is £4 per volume, payable in advance to the Cambridge University Press, Bentley House, 200 Euston Road, London, N.W. 1, or to any bookseller. In the United States of America the *Journal* may be purchased from the Cambridge University Press American Branch, 32 East 57th Street, New York, 22 (subscription price $13.50 per volume).

Communications respecting the sale of single issues or back numbers of the *Journal*, Volumes 20–73, should be addressed to the Cambridge University Press, Bentley House, 200 Euston Road, London, N.W. 1, from whom a list of prices is available. Volumes 1–15 have been reproduced and are available from William Dawson & Sons, Ltd., 4 Duke Street, Manchester Square, London, W. 1.

Claims for the replacement of *Journals* lost in transmission will not be entertained if they are received later than three months after the date of the posting of the *Journal*.

The following are available in paper covers:

Volumes 20–28.
Volume 29 Nos. 1, 2, 4, 6, 8–11.
Volume 33 Nos. 8–12.
Volume 34 Nos. 4–7 & 12.
Volume 34 Nos. 8 & 9, 10 & 11.
Volumes 35–38.
Volume 39 Nos. 2 & 3.
Volumes 40–47.
Volumes 50–58.
Volumes 60–73.
Index to Volumes 11–20.
Index to Volumes 21–30.
Index to Volumes 31–40.
Index to Volumes 41–65.
History of the Biochemical Society.

Reduced terms for Volumes 1–15 are available to members of the Biochemical Society, who should apply to the Honorary Secretaries.

The Separation of Nitrogen-Containing Phosphate Esters from Brain and Spinal Cord by Ion-Exchange Chromatography

By R. W. R. BAKER and G. PORCELLATI*
Department of Chemical Pathology, Guy's Hospital Medical School, London, S.E. 1

(*Received* 21 *April* 1959)

The quantitative behaviour of ion-exchange resins in the analysis by column chromatography of mixtures of amino acids (Moore & Stein, 1951) is already well established. Microbead sulphonated polystyrene resins have been used for analysing protein hydrolysates (Askonas, Campbell, Godin & Work, 1955; Van der Schaaf & Huisman, 1955; Jacobs, 1956*a*). Similar methods were employed in the study of phosphorus-containing peptides present in hydrolysed protein by Flavin (1954) and by Schaffer, Harshman & Engle (1955); Tallan, Moore & Stein (1954) used them for separating phosphorylethanolamine and glycerylphosphorylethanolamine from tissue extracts. Apart from the last-mentioned work the technique has, however, been applied but little in the study of nitrogen-containing phosphate esters, and in particular no report on the analysis by this means of phosphorylcholine and its derivatives has yet appeared.

In a study of protein-free aqueous extracts of hen brain and spinal cord we have characterized some of the peaks obtained by chromatography and have compared the two tissues. This paper is concerned primarily with peaks containing both nitrogen and phosphorus. In addition to analysis of fractions of eluate by the ninhydrin reaction, they were analysed for phosphorus and choline. Glycerylphosphorylcholine and phosphorylcholine were distinguished by the fact that the former is rapidly hydrolysed by acid, whereas the latter requires more vigorous conditions.

The presence of phosphorylserine, glycerylphosphorylethanolamine and phosphorylethanolamine in brain and spinal cord, and of phosphorylcholine, glycerylphosphorylcholine and of other phosphorus compounds in brain, has been confirmed; amounts of some of these have been measured. The chromatographic behaviour of some free phosphate esters has been studied quantitatively.

EXPERIMENTAL

Materials

Ion-exchange resin. Zeo-Karb 225, 4·5% cross-linked (water-regain 1·8 g./g.), 200–400 mesh, (The Permutit Co. Ltd.) was washed with 10 vol. of water and graded by stirring with water (5 vol.) and allowing about 5% (v/v) of the resin to settle. The remaining suspension was decanted and 95% of the solid allowed to settle out in about 15 hr.; the cloudy supernatant was discarded. The portion of the resin selected corresponded to 85–90% (v/v) of the original material. Dr S. Jacobs kindly examined the resulting resin and reported a range in bead diameter of 10–50 μ. It was converted into the sodium form by treatment with 2N-NaOH (2 vol.), separated by filtration under reduced pressure and washed free of excess of alkali with five changes of water.

For use, the resin was washed with 0·5N-NaOH and then equilibrated with 0·2M-sodium citrate buffer, pH 5·1 (see below), by suspending twice in 3 vol. followed by sedimentation and rejection of the supernatant liquid.

Detergent. The polyoxyethylene lauryl ether BRIJ 35 (L. Light and Co. Ltd.) was used in some experiments.

Thiodiglycol. This compound (British Drug Houses Ltd.) was redistilled under reduced pressure, the middle third of the distillate (b.p. 168°/14 mm.) being collected.

Buffer solutions. (1) Sodium citrate, 0·2M, pH 2·2: citric acid 42 g.; NaOH 16·8 g.; conc. HCl 32 ml.; to 2 l. with water. (2) Sodium citrate 0·2M, pH 3·1: citric acid 71·4 g.; NaOH 28·2 g.; conc. HCl 39·3 ml.; to 3·4 l. with water. (3) Sodium citrate 0·2M, pH 5·1: citric acid, 4·2 g., N-NaOH 40 ml.; to 200 ml. with water.

pH values were measured with a glass electrode. Each buffer contained 0·5% of thiodiglycol and 0·01% of detergent, and was stored at 3°, but in experiments in which fractions were to be hydrolysed with HCl the thiodiglycol was omitted (Hirs, Moore & Stein, 1956). No detergent was used in experiments in which choline was estimated, since the former interfered in the iodine method.

Methods

Chromatographic methods. Buffered resin, suspended in its own volume of buffer solution (3), was poured in four portions into a vertical glass tube (12·5 mm. bore) fitted with a sintered-glass diaphragm at its lower end to give a column 166 cm. long. The column settled under gravity to a volume of 210 ml., as solution ran off through the sintered glass until the liquid was level with the top of the resin. Before applying the sample material, 0·2N-NaOH (100 ml.), followed by at least 250 ml. of buffer (1), was passed through under a pressure (20 cm. Hg) of nitrogen. Both solutions generally contained detergent and thiodiglycol as above. When the liquid was down to bed level, the sample (2–3 ml. adjusted to pH 2·0–2·5) was poured on and drained to bed level. Fractionation of the mixture was then carried out at 22° by passing citrate buffer (2) under a pressure (20–25 cm. Hg) of nitrogen, the arrangement being shown in Fig. 1. Effluent (about 500 ml.) was

* British Council Scholar.

collected in 2 ml. fractions, the flow rate being 5 ml./hr. The column was then regenerated *in situ* with NaOH and buffer (1) as described above.

For examination of peaks detected as described below, the component fractions were pooled and two-dimensional chromatography on paper was applied as reported earlier (Porcellati, 1958). Where necessary, removal of cations from solutions (Jacobs, 1956*b*) was carried out by passing them under pressure (10 cm. Hg.) of nitrogen through columns (5 cm. long, 1 cm. diam.) of Zeo-Karb 225 microbead resin in the free-acid form, washed *in situ* with water. After adsorption, the column was washed free of acids with water and then eluted with aq. 0·2N-NH_3 soln. (45 ml.). Eluates were distilled under reduced pressure to 2–3 ml. and evaporated to dryness on a water bath. Residues were taken up in 0·1N-HCl for application to paper. Washing of the resin before and after adsorption was controlled by measuring the conductivity of effluent with an alternating-current bridge and platinized electrodes; generally 50 ml. of water was required for each wash.

Analysis of effluent. (1) *Ninhydrin reaction.* The method of Moore & Stein (1954), modified as described below, was used with all or part of each fraction tested, water being used to bring the sample to 2 ml. As standard, leucine (0·2 μmole/2 ml. in citrate buffer, pH 5·1) was used. The original preparation (Moore & Stein, 1954) of the ninhydrin–hydrindantin reagent was modified (Jacobs, 1956*b*) in that the solution of ninhydrin (AnalaR) and hydrindantin in methyl Cellosolve (2-methoxyethanol) was treated with its own volume of Zeo-Karb 225 (H) to remove ammonia and trace metals and stored over the resin in an atmosphere of nitrogen before mixing with acetate buffer. Blank values were reduced to a uniform low value as a result.

(2) *Choline.* After hydrolysis of 1 ml. from the fraction in 2N-HCl for 1 hr. under reflux, or for 48 hr. in a sealed tube at 123°, the solution was evaporated to dryness on a water bath, with further evaporation, after addition of 3 ml. of water, to remove HCl. The residue was dissolved in 5% (w/v) trichloroacetic acid and choline was determined by the periodate method (Webster, 1956).

(3) *Phosphate.* The method of Ernster, Zetterström & Lindberg (1950) as modified by Strickland, Thompson & Webster (1956) was used.

Tissue extracts. Hens of mixed breed were decapitated and the brains and spinal cords were removed quickly and cooled on ice. Weighed amounts of tissue, finely minced and homogenized in 2–3 vol. of ice-cold water, were deproteinized by bringing to pH 4·8–5·0 with 0·5N-HCl and pouring into 20 vol. of boiling water. The precipitate, filtered off after 2–3 min., was washed twice with hot water. The combined filtrate and washings were concentrated under reduced pressure to about 10 ml. and dialysed at 4° for two 12 hr. periods against 100 ml. of water with continuous agitation. The combined dialysates were concentrated to 2–3 ml. under reduced pressure, adjusted to pH 2·0–2·5 with 0·1N-HCl and applied to the ion-exchange column. The extract applied corresponded to 2·0–2·5 g. of fresh tissue.

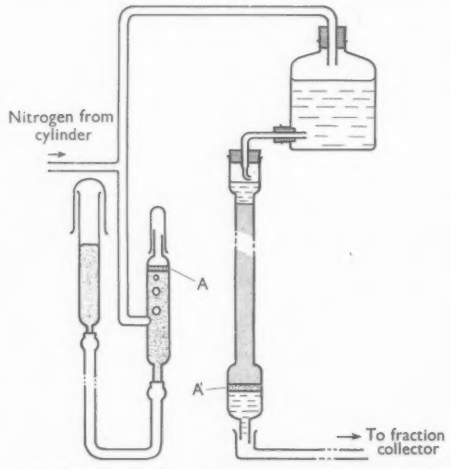


Fig. 1. Apparatus for ion-exchange chromatography (schematic). A and A′ are diaphragms of sintered glass.

RESULTS

The results of chromatography of extracts of brain and cord are shown in Figs. 2 and 3, where the extinctions after reaction with ninhydrin, calculated as μmoles of leucine/2 ml. of fraction, are plotted against the serial numbers of the fractions taken. After identification of some of the peaks, the colour yields with ninhydrin of the substances involved were determined and approximate closely to published data (Moore & Stein, 1954). This enabled the absolute amounts of the substances to be calculated (Table 1). Collection of the eluate was continued to a total volume of about 500 ml., since only the relatively acidic phosphate esters were being studied. Figs. 2 and 3 give a direct comparison of brain with spinal cord. The latter gave, in fractions 108–111, 113–118 and 146–148, minor constituents which were not found in the brain, whereas a small fraction (33–36) in brain was not found in the cord; none of these fractions was examined since insufficient material was obtained. The other differences observed are in the quantities present, and in particular the fraction 138–141, abundant in cord, is a relatively small component of brain (135–139).

Preliminary chromatography on a shorter column (140 cm.) of a mixture of three amino acids had shown, as discussed below, that our fraction numbers corresponded closely with the eluate volumes reported by Tallan *et al.* (1954), and that recoveries were quantitative. Provisional identification of our peaks was therefore made by reference to the results of these authors, and suitable fractions were chosen for further study. They were desalted with ion-exchange resin and examined by paper chromatography by the methods described above.

Analysis for phosphorus and examination of the materials by paper chromatography, both as obtained and after hydrolysis, showed that peak

Table 1. *Colour yields with ninhydrin and amounts of certain compounds in avian nervous tissue*

Substance	Colour-yield/μmole (relative to leucine = 100)	Amounts in protein-free aqueous extracts of avian nervous tissue (mg./100 g. of fresh tissue)							
		Brain				Spinal cord			
		No. of determinations	Range	Mean	S.D.	No. of determinations	Range	Mean	S.D.
Phosphorylserine	74	9	0·6–2·3	1·36	0·56	4	0·6–1·2	0·95	0·26
Phosphorylethanolamine	44	10	7·5–31·8	19·6	0·60	4	0·6–7·8	6·9	0·79
Glycerylphosphoryl-ethanolamine	52	9	11·6–26·0	17·1	5·46	4	12·3–14·1	13·1	0·83
Taurine	90	9	14·3–30·7	18·5	5·18	4	4·4–7·1	6·1	1·23
Aspartic acid	95	9	27·1–53·8	38·6	9·50	2	24·1–29·6	26·9	—
Threonine	94	8	6·1–11·2	7·8	1·62	2	5·8–6·2	6·0	—
Glycerylphosphoryl-choline	0	10	1·9–7·9	5·2	2·08	3	1·9–4·8	3·2	1·47
Phosphorylcholine	0	9	12·0–17·2	14·1	1·83	3	7·0–8·1	7·5	0·56

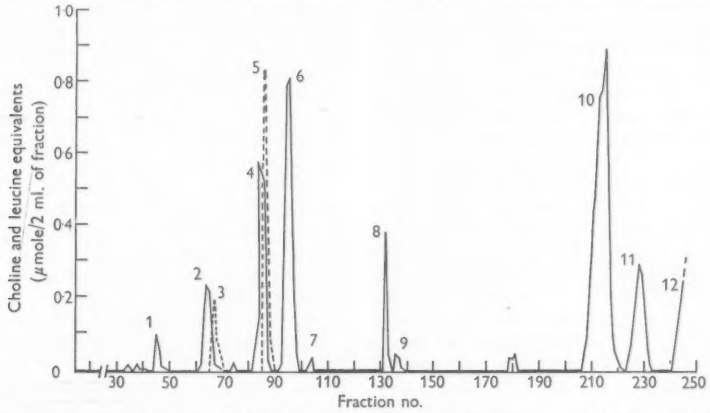


Fig. 2. Chromatogram of extract from 2·5 g. of hen brain. —, Leucine equivalent; - - - -, choline in hydrolysed fractions. 1, Phosphorylserine; 2, glycerylphosphorylethanolamine; 3, glycerylphosphorylcholine; 4, phosphorylethanolamine; 5, phosphorylcholine; 6, taurine; 7, urea; 8, unknown; 9, peptide; 10, aspartic acid; 11, threonine; 12, serine.

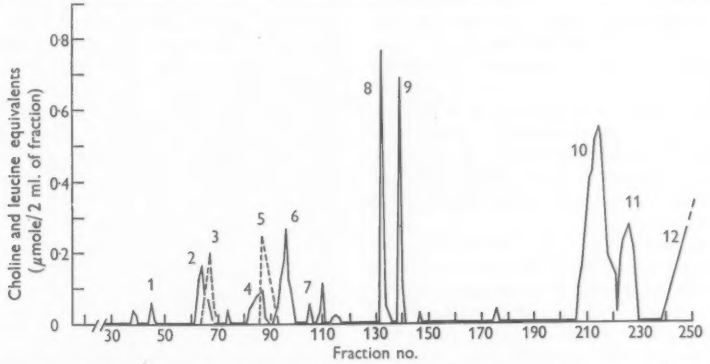


Fig. 3. Chromatogram of extract from 2·1 g. of hen spinal cord. —, Leucine equivalent; - - - -, choline in hydrolysed fractions. For significance of numbered peaks see caption to Fig. 2.

45–49 (44–46 in cord) was phosphorylserine. The molar ratio amino nitrogen:P was 1·10 (calc. 1·00). Peak 61–71 (61–69 in cord) was identified as glycerylphosphorylethanolamine by chromatography of the substance alone and after admixture with the authentic material. Chromatograms of the hydrolysed materials (2N-HCl; 2 hr.; 100°) revealed the presence of glycerophosphate, ethanolamine and a small amount of inorganic phosphate. The molar ratio amino nitrogen:P was 0·89, a low value which may be interpreted as due to contamination of the substance with glycerylphosphorylcholine.

The major component 82–87 (82–88 in cord) was found to be phosphorylethanolamine. After hydrolysis (6N-HCl; 48 hr.; 120°; sealed tube), evaporation and desalting, analysis on paper chromatograms showed the presence of ethanolamine, inorganic phosphate and a small amount of unchanged phosphorylethanolamine. The molar ratio amino nitrogen:P found was 0·98 for the peak material. The unidentified minor peak (33–36) was of high phosphorus content, amino nitrogen and P being present in approximately equimolar amounts.

Brain fractions (37–41) gave after hydrolysis a deeper colour with ninhydrin, and on paper chromatography six ninhydrin-reacting spots were revealed, and this peak is therefore considered to be of peptide-like composition; no phosphorus was detected. Another component (fractions 135–139 in brain and 138–141 in cord) showed similar behaviour but contained phosphorus. The identity of these peaks was confirmed by paper chromatography with and without hydrolysis, by analysis for phosphorus and by the pentose reaction with orcinol. Although in brain the quantity of this fraction is such that only 44 μg. of P is present in 100 g. of wet tissue, the amount in spinal cord is very much larger (more than 0·7 mg. of P/100 g. of tissue). The substance was hydrolysed only slowly in 6N-HCl (sealed tube; 120°) and was still detectable after 48 hr. The absorption found in the ninhydrin reaction with hydrolysate was about 6·4 times that obtained with the parent peptide. Among the five or six major products of hydrolysis resolved on paper chromatograms, provisional identifications of phosphorylserine, phosphorylethanolamine, glutamic acid and valine have been made. The original substance gave a green in the orcin reaction for pentose.

In addition, some of the more prominent ninhydrin-reacting fractions from brain not containing P were identified as taurine (91–100), aspartic acid (206–222), threonine (223–233) and serine (241 *et seq.*). The prominent phosphorus-free component (fractions 131–133) and a lesser one (178–182) remain unidentified.

The presence of phosphate esters of choline in the brain extract was confirmed in further work with similar columns. Fractions of eluate were analysed by hydrolysing and then determining the choline liberated. This was necessary since choline, in the quantities treated, does not give any colour with ninhydrin, a fact that confers some specificity. Two fractions containing choline were found (Fig. 2). The peak (65–71) liberated all its choline after hydrolysis (6N-HCl; 100°) for 1 hr., as is found with glycerylphosphorylcholine, in contrast with phosphorylcholine, which is hydrolysed only under the more vigorous conditions described below (Dawson, 1955). The position of the peak corresponded with that found when authentic glycerylphosphorylcholine was run chromatographically in the same way. The pooled fractions of the peak from brain extract, after desalting, were examined by paper chromatography in two directions, with spraying to reveal esterified phosphate (Bandurski & Axelrod, 1951); the result confirmed the identity of the glycerylphosphorylcholine and revealed the presence in the same fraction of glycerylphosphorylethanolamine, as had been found earlier. Control experiments showed that the latter, in a quantity such as that present, did not influence the determinationation of choline by the method used, although excess of glycerylphosphorylethanolamine (2 mg.), when hydrolysed as described above, gave a faint final magenta pink in $CHCl_3$, clearly different from the red–orange given by choline. Since the two substances are resolved poorly if at all by the column, analysis for P is equivocal.

The second peak in which choline was detected (85–91, Fig. 2) was identified as phosphorylcholine, which yields the base quantitatively only on hydrolysis for 48 hr. (6N-HCl; 120°; sealed tube). Two-dimensional chromatograms of the unhydrolysed pooled desalted material, sprayed to reveal esterified phosphorus, showed a spot corresponding to that for authentic phosphorylcholine. The choline:P molar ratio was 1·04:1. Chromatography on the column of pure phosphorylcholine gave a peak in the same position, revealed by analysis both for P and for choline. Our result indicates 12·0–17·2, mean 14·1 mg./100 g., as the phosphorylcholine content of brain. Taurine, urea, HCl and citrate buffer were shown in control experiments not to interfere with the estimation of esterified choline by the hydrolysis and colorimetric methods employed. Recoveries of 98 and 99% for phosphorylcholine and glycerylphosphorylcholine respectively were recorded when a mixture of 2 μmoles of each of the pure compounds was run on the column, followed by estimation of both P and choline in the effluent. No free choline was detected in the eluates examined, as would be expected since such a basic substance would appear only at much greater eluate volumes.

Hen-brain extract from one bird only was analysed directly for choline compounds, without acid treatment and after hydrolysis for 1 hr. or 48 hr. as described above (Dawson, 1955). This differential hydrolysis indicated that 100 g. of the tissue contained 2·4 mg. of 'free choline', 2·1 mg. of glycerylphosphorylcholine and 9·8 mg. of phosphorylcholine. The value for free choline obtained by the iodine method with an extract containing so many substances should be taken only as provisional.

DISCUSSION

The chromatographic method described appears to be well suited, in conjunction with specific colorimetric methods, for the determination not only of amino acids, but of phosphate esters of choline, ethanolamine and serine in extracts of brain or spinal cord. Sharp peaks were obtained with each of the various classes of substance, ranging from those of relatively small molecular weight to others of grosser structure resembling peptides. Some overlapping did occur, as, for example, with the glycerylphosphoryl derivatives of ethanolamine and choline, but here confusion was avoided by the differential use of specific methods of analysis.

The value obtained by Tallan *et al.* (1954) by column chromatography for the amounts of phosphorylethanolamine (41·9 mg./100 g.) in cat-brain extracts is greater than the quantities found in rat brain by other authors (12 mg./100 g., Awapara, Landua & Fuerst, 1950; 9 mg./100 mg., Ansell & Dawson, 1952; 22 mg./100 mg., Ansell & Richter, 1954) who relied on paper chromatograms. With hen brain, there was reasonable agreement found in this Laboratory between the values from the column and results with paper chromatography. Thus for phosphorylethanolamine a quantity of 19·6 mg./100 g. of brain was found with the column, and 13·8 mg./100 g. by the paper method (Porcellati, 1958). For phosphorylcholine also the results with the column (12·0–17·2 mg./100 g.) are fairly close to those obtained by paper chromatography (9·2 mg./100 g., Porcellati, 1958). These values quoted from analysis on papers have not been corrected for the loss that is known to occur. Whereas recoveries in two-dimensional chromatography are known to be between 75 and 85%, the column analysis is much more quantitative, as shown, for example, by recoveries of 17 amino acids (Moore & Stein, 1954), and as found in the present work with phosphate esters of choline. Lower quantities of phosphorylserine were, however, found in brain by the column method (1·36 mg./100 g.) than by paper chromatography (4·3 mg./100 g.) (Porcellati, 1958). This discrepancy can probably be attributed to the superior resolving power of the column, for on the paper the phosphorylserine may be contaminated with peptides.

Since this paper was written, there has appeared a report (Okumura, Otsuki & Aoyama, 1959) of analysis of hen-brain extract on Dowex resin. Values found for glycerylphosphorylethanolamine (24·9 mg./100 g. of tissue) and aspartic acid (33·4 mg./100 g.) fall within our range; values for phosphorylethanolamine (45·8 mg./100 g.) and taurine (4·4 mg./100 g.) are higher whereas the amount of threonine (4·2 mg./100 g.) is lower than that found in the present investigation.

SUMMARY

1. Deproteinized aqueous extracts of avian brain and spinal cord have been resolved by chromatography on a column of a finely divided sulphonated polystyrene resin and the more mobile components examined.

2. The method is shown to be suitable for the separation and quantitative estimation of phosphorylcholine and glycerylphosphorylcholine in brain and spinal cord.

3. Phosphorylserine was well resolved. Phosphorylethanolamine and glycerylphosphorylethanolamine, although overlapping to some extent their choline counterparts, could be determined by specific reaction with ninhydrin and the quantities present in brain were ascertained.

The authors wish to thank: Professor R. H. S. Thompson for his interest and provision of facilities; Dr G. R. Webster for gifts of phosphorylserine, phosphorylethanolamine, phosphorylcholine and glycerylphosphorylcholine; Dr R. M. C. Dawson for a gift of glycerylphosphorylethanolamine and Dr S. Jacobs for helpful discussion on the methods and on the properties of the resin used. They are also grateful to the British Council for a grant to one of us (G.P.).

REFERENCES

Ansell, G. B. & Dawson, R. M. C. (1952). *Biochem. J.* **50**, 241.

Ansell, G. B. & Richter, D. (1954). *Biochem. J.* **57**, 70.

Askonas, B. A., Campbell, P. N., Godin, C. & Work, T. S. (1955). *Biochem. J.* **61**, 105.

Awapara, J., Landua, A. J. & Fuerst, R. (1950). *J. biol. Chem.* **183**, 545.

Bandurski, R. S. & Axelrod, B. (1951). *J. biol. Chem.* **193**, 405.

Dawson, R. M. C. (1955). *Biochem. J.* **60**, 325.

Ernster, L., Zetterström, R. & Lindberg, O. (1950). *Acta chem. scand.* **4**, 942.

Flavin, M. (1954). *J. biol. Chem.* **210**, 771.

Hirs, C. H. W., Moore, S. & Stein, W. H. (1956). *J. biol. Chem.* **219**, 623.

Jacobs, S. (1956*a*). *Rés. Comun.* 11^e *Congr. Quim. anal., Lisbon*, p. 212.

Jacobs, S. (1956*b*). *Analyst*, **81**, 502.
Moore, S. & Stein, W. H. (1951). *J. biol. Chem.* **192**, 663.
Moore, S. & Stein, W. H. (1954). *J. biol. Chem.* **211**, 907.
Okumura, N., Otsuki, S. & Aoyama, T. (1959). *J. Biochem., Tokyo*, **46**, 207.
Porcellati, G. (1958). *J. Neurochem.* **2**, 128.
Schaffer, N. K., Harshman, S. & Engle, R. R. (1955). *J. biol. Chem.* **214**, 799.
Strickland, K. P., Thompson, R. H. S. & Webster, G. R. (1956). *J. Neurol. Psychiat.* **19**, 12.
Tallan, H. H., Moore, S. & Stein, W. H. (1954). *J. biol. Chem.* **211**, 927.
Van der Schaaf, P. C. & Huisman, T. H. J. (1955). *Biochim. biophys. Acta*, **17**, 81.
Webster, G. R. (1956). *Biochim. biophys. Acta*, **20**, 432.

The Phosphate Esters of Mammalian Skin Maintained on Glucose and Various Deoxyglucoses

By SHEILA A. BROOKS, J. C. LAWRENCE and C. R. RICKETTS
Medical Research Council Industrial Injuries and Burns Research Unit, Birmingham Accident Hospital, Birmingham, 15

(*Received* 23 *April* 1959)

In most living cells the first step in glycolysis is phosphorylation of glucose at C-6, catalysed by the enzyme hexokinase. Studies with hexokinase preparations from yeast and from brain show that compounds closely related to glucose may also be phosphorylated, e.g. 1:5-anhydro-D-glucitol, 2-deoxy-D-glucose, D-glucosone, D-glucosamine and D-mannose (Harpur & Quastel, 1949; Brown, 1951; Johnstone & Mitchell, 1953; Sols & Crane, 1954; Woodward & Hudson, 1955), but 3-deoxy-D-glucose, *N*-acetyl-D-glucosamine and D-galactose are not phosphorylated (Grant & Long, 1952; Sols & Crane, 1954). Of these compounds 2-deoxyglucose, glucosone and glucosamine are known to interfere with tumour-cell glycolysis (Woodward & Hudson, 1954; Yushok & Batt, 1957). 2-Deoxyglucose is also reported to inhibit the synthesis of influenza virus in chick embryos (Kilbourne, 1959), presumably by functioning as an antimetabolite of glucose. Wick, Drury, Nakada & Wolfe (1957) concluded that the inhibition of glycolysis which occurred when 2-deoxyglucose was given to rabbits was at the phosphoglucoisomerase stage of glycolysis. Other than this there seems to be very little published information about the effects of deoxysugars on normal mammalian cells and further experiments seemed desirable. Tissue culture affords a convenient approach to this problem and in the experiments to be described slices of mammalian skin were maintained on media containing 1:5-anhydro-D-glucitol (which may be regarded as 1-deoxy-D-glucose), 2-deoxy-D-glucose and 3-deoxy-D-glucose (Fig. 1). D-Glucosone, D-mannose and D-glucosamine were also included since these hexoses differ from glucose at C-2.

The metabolism of the skin cells has been studied by micro-respirometry and by chromatography and radioautography of the phosphate esters formed during maintenance on radioactive inorganic phosphate. It was first necessary to identify the phosphate esters of skin maintained on glucose and then to examine skin maintained on deoxysugars. For this purpose certain phosphate esters

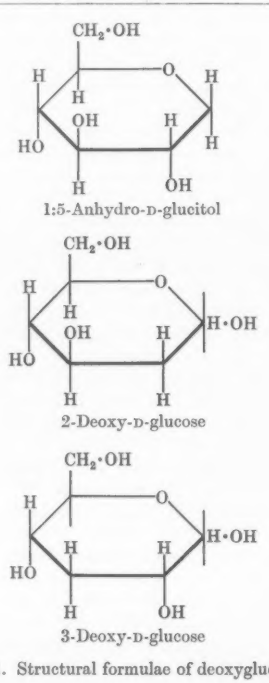


Fig. 1. Structural formulae of deoxyglucoses.

were prepared by the action of yeast hexokinase and used to identify the corresponding [^{32}P]esters produced by skin cells.

EXPERIMENTAL

Materials

1:5-*Anhydro*-D-*glucitol*. This was prepared by the reduction of 2:3:4:6-tetra-*O*-acetyl-1-bromo-α-D-glucopyranoside with lithium aluminium hydride (Ness, Fletcher & Hudson, 1950). The product had m.p. 142° and $[\alpha]_D^{20}$ +42·4°.

2-*Deoxy*-D-*glucose*. This was prepared from glucose via 3:4:6-tri-*O*-acetyl-D-glucal (Overend, Stacey & Staněk, 1949). The triacetylglucal was recrystallized several times to ensure that the final product was free from glucose. The product had m.p. 148° and $[\alpha]_D^{20}$ +46·6°. No glucose could be detected in a 15% (w/v) solution subjected to paper electrophoresis.

3-*Deoxy*-D-*glucose*. This was obtained by hydrolysing 3-deoxy-1:2-5:6-di-*O*-*iso*propylidene-α-D-glucose (kindly supplied by Dr E. J. Hedgley) in water at 100° with an ion-exchange resin.

D-*Glucosone*. This was kindly provided by Dr S. Bayne. This substance, which is extremely difficult to purify, was available as a syrup which was freeze-dried to facilitate weighing. Electrophoresis in borate buffer on paper (Foster & Stacey, 1955) and paper chromatography (phenol–water; 4:1) showed that the major constituent M_G 0·95 and R_G 0·74 was accompanied by traces of two other substances M_G values 0·38 and 0·65, R_G values 1·65 and 1·91. (M_G applies to electrophoresis and is the distance migrated by the substance divided by the distance migrated by glucose, the distances being corrected for endosmotic flow. R_G applies to chromatography and is the distance moved by the substance divided by the distance moved by glucose.) No glucose could be detected even when a 15% (w/v) solution was applied to the papers and therefore this sample was considered suitable for metabolic experiments.

N-Acetyl-D-*glucosamine*. This compound, m.p. 206° (decomp.), was prepared by acetylation of D-glucosamine (Pierce & Kane, 1954).

Phosphate esters. 1:5-Anhydro-D-glucitol, 2-deoxy-D-glucose, D-mannose, D-glucosone and glucosamine were each phosphorylated with yeast hexokinase and adenosine triphosphate (ATP). The resulting phosphate ester was separated by fractional precipitation of the barium salts with ethanol, as described by Brown (1951) for the preparation of glucosamine 6-phosphate. The esters from 1:5-anhydro-D-glucitol and 2-deoxyglucose showed the same R_F value when chromatographed on paper in propan-1-ol–aq. NH_3 soln. as the chemically synthesized esters (Brooks, 1959). The following were also used as reference substances on chromatograms: dipotassium-α-D-glucose 1-phosphate dihydrate ($[\alpha]_D$ +78·8°), prepared from starch by the action of potato phosphorylase, D-glucose 6-phosphate, D-fructose 6-phosphate, D-fructose 1:6-diphosphate and 3-phospho-D-glyceric acid (British Drug Houses Ltd.), adenosine mono-, di- and tri-phosphate (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) and uridine diphosphoglucose, guanosine di- and tri-phosphate, uridine di- and tri-phosphate and phosphoenolpyruvic acid (Sigma Chemical Co., St Louis, Mo., U.S.A.). Where necessary, barium salts were converted into the corresponding sodium salts before use.

Methods

Tissues and media. Slices of guinea-pig skin were obtained from the ear of the adult animal as described by Cruickshank (1954).

The basal medium was Krebs–Ringer phosphate (Field, 1948) containing 50 μg. of streptomycin sulphate (Glaxo Laboratories Ltd.)/ml. Substrates were prepared at 0·2M in 0·85% NaCl and sterilized by filtration through sintered glass (porosity no. 5; maximum pore size < 1·0 μ).

Respiration studies. The differential microrespirometer described by Cruickshank (1954) was used for respiration studies. This instrument enables the respiration of small quantities of skin to be measured accurately for periods of up to 3 days. Between 8 and 11 mg. of skin floating on 0·45 ml. of Krebs–Ringer phosphate was assigned to each respirometer and the oxygen uptake measured for 1·5 hr. at 37°, the gas phase being air. Substrate (0·05 ml.) was then added by means of a Pasteur pipette, making a 1:10 dilution with the basal medium and giving a final substrate concentration of 0·02M. For experiments with no substrate 0·05 ml. of 0·85% NaCl was added. Respiration measurements were then continued for a further 4–5 hr.; they were discontinued overnight (about 16 hr.) and were recommenced the following day and continued up to 24 hr. after addition of the substrate. All changes in respiratory rate due to substrate were then related to the initial 1·5 hr. control period without substrate.

No measurements of pH changes were made although Cruickshank (1954) quotes pH values between 8·5 and 8·0 during respiration experiments over periods of 24 hr.

Phosphate ester studies. For each experiment about 20 mg. of skin was maintained on Krebs–Ringer phosphate containing 0·15 mC of [^{32}P]orthophosphate in 10 ml. It was found best to distribute the skin among three or four containers each holding 0·45 or 0·9 ml. Substrate solution (or medium only) was added to make a final concentration of 0·02M and the systems were incubated for 24 hr. at 37°. After this time sterility was checked by streaking a loopful of the medium across a blood–agar plate; this was incubated for 1 day. The skin was ground in a hand-operated glass tissue-grinder (A. Gallenkamp and Co. Ltd.) in a minimal quantity of water. Solid material was removed by centrifuging and the protein was precipitated by addition of one drop of trichloroacetic acid (100%, w/v). The precipitate was removed by further centrifuging and the trichloroacetic acid neutralized by addition of aq. NH_3 soln. [sp.gr. 0·880 diluted 1:1 (v/v) with water]. Approximately 5 mg. of ethylenediaminetetra-acetic acid (EDTA) was then added and the resultant solution (about 0·1 ml.) used for chromatography; this was stored at −20°. Extracts from skin contaminated with bacteria or fungi were discarded.

Chromatography. Two-dimensional chromatography based on the method of Hanes & Isherwood (1949) was used. Sheets of Whatman no. 1 filter paper (18½ in × 22½ in.) were soaked overnight in 0·2% EDTA adjusted to pH 7·8 with NaOH. They were then washed several times with water and allowed to dry at room temperature.

About 10 spots of skin extract were successively dried on the paper (in certain experiments solutions of reference substances were mixed with the skin extract at this stage), and chromatographed in propan-1-ol–aq. NH_3 soln. (sp.gr. 0·880)–water (60:30:10, by vol.) for 40 hr. The solvent front had run well clear of the paper after this time. The

paper was dried, rotated through 90° and chromatographed in picric acid–2-methylpropan-2-ol–water (4:80:20, w/v/v) for 24 hr. The solvent front was usually about 2 in. from the bottom of the paper.

Phosphate esters were present in amounts too small for detection by chemical means and only inorganic phosphate was detected by spraying. Radioautographs were prepared by attaching the chromatogram to the packet containing a Kodirex X-ray film (14 in. × 17 in.) by means of drawing pins at the origin and two corners. An exposure of 9 days was found adequate for an extract prepared from 20 mg. of skin cultured on media containing about 15 μc of [^{32}P]/ml. on commencing the exposure. Weaker extracts were given correspondingly longer exposures calculated from the radioactive decay.

Reference compounds were detected by spraying with a solution containing 5 ml. of 60% (w/w) perchloric acid, 10 ml. of N-HCl, 25 ml. of 4% (w/v) ammonium molybdate and water to 100 ml., and heating at 85° for 7 min. (Hanes & Isherwood, 1949). The paper was then sprayed with 5% (w/v) ascorbic acid and phosphate esters appeared as blue spots (Leuthardt & Testa, 1951). The chromatogram and radioautograph were placed together, the pin-holes being used to locate the two, and the blue area was traced on to the film thus forming a permanent record. If the marker compound was present in the skin extract one of the areas of blackening coincided with the blue coloration.

Because of their resistance to acid hydrolysis some hexose 6-phosphates cannot be readily detected by the molybdate reagent. Such esters were demonstrated by spraying with aniline hydrogen phthalate; this process does not interfere with subsequent detection of other phosphate esters by the molybdate reagent.

RESULTS

Respiration on hexoses and deoxyglucoses

It was found that skin maintained without substrate respired steadily for the first 2 hr., presumably utilizing endogenous substrate, after which time the respiration rate fell gradually. If the 0–1·5 hr. period is taken as 100%, the next 2-hr. period shows a decrease of about 15% and the 22–24 hr. period a decrease of about 65%. If glucose (0·02 M) was added after 1·5 hr. the respiration rate was still at the initial rate 4 hr. later and decreased by only 18% after 24 hr. These findings are in agreement with the observations made by Cruickshank, Trotter & Cooper (1957).

It was found that mannose (0·02 M) and galactose (0·02 M) were able to support the respiration of skin to the same extent as glucose (Table 1). Though the percentage of the original value for skin maintained in galactose is lower than that for glucose (77 and 84% respectively) the difference is not statistically significant for the number of experiments carried out.

Glucosamine (0·02 M) also supported skin respiration, but not as efficiently as glucose, respiratory survival being 56%, a significantly lower amount. Glucosone and 1:5-anhydro-D-glucitol could also support skin respiration to a similar extent (about 50% survival after 24 hr.). However, these two

Table 1. *Respiration of guinea-pig skin on various substrates*

Respiratory rates are given ± S.E.M.

Substrate (concn. 0·02 M)	No. of experiments	Uptake of oxygen: 0–1½ hr. before substrate was added (μl./mg. wet wt./hr.)	0–2 hr. after substrate was added (μl./mg. wet wt./hr.)	Percentage of initial rate	22–24 hr. after substrate was added (μl./mg. wet wt./hr.)	Percentage of initial rate
None	9	0·70 ± 0·02	0·60 ± 0·02	86	0·23 ± 0·01	33
D-Glucose	11	0·69 ± 0·03	0·69 ± 0·04	100	0·58 ± 0·03	84
1:5-Anhydro-D-glucitol	4	0·63 ± 0·01	0·71 ± 0·02	113	0·35 ± 0·05	56
1:5-Anhydro-D-glucitol + D-glucose	4	0·68 ± 0·06	0·73 ± 0·06	107	0·54 ± 0·05	79*
2-Deoxy-D-glucose	6	0·69 ± 0·02	0·56 ± 0·03	81	0·21 ± 0·03	30†
2-Deoxy-D-glucose + D-glucose	4	0·66 ± 0·08	0·58 ± 0·09	88	0·31 ± 0·05	47
3-Deoxy-D-glucose	5	0·71 ± 0·02	0·62 ± 0·02	87	0·25 ± 0·03	35†
3-Deoxy-D-glucose + D-glucose	5	0·63 ± 0·03	0·62 ± 0·03	98	0·54 ± 0·04	86*
D-Glucosone	5	0·66 ± 0·02	0·79 ± 0·07	120	0·30 ± 0·03	45
D-Glucosone + D-glucose	5	0·61 ± 0·02	0·69 ± 0·05	113	0·50 ± 0·02	82*
D-Glucosamine	8	0·72 ± 0·03	0·71 ± 0·04	99	0·40 ± 0·06	56
D-Glucosamine + D-glucose	3	0·68 ± 0·07	0·66 ± 0·07	97	0·57 ± 0·06	84*
N-Acetyl-D-glucosamine	6	0·65 ± 0·01	0·65 ± 0·02	100	0·22 ± 0·04	34†
N-Acetyl-D-glucosamine + D-glucose	3	0·65 ± 0·05	0·62 ± 0·08	95	0·51 ± 0·06	78*
D-Mannose	11	0·71 ± 0·03	0·70 ± 0·04	99	0·57 ± 0·03	80*
D-Galactose	8	0·68 ± 0·03	0·69 ± 0·04	102	0·52 ± 0·06	76*
D-Galactose + 2-deoxy-D-glucose	3	0·70 ± 0·02	0·69 ± 0·02	99	0·57 ± 0·02	81*

* Not significantly different from glucose.
† Not significantly different from 0·85% NaCl.

substances differed markedly from all the others tested by causing a significant initial increase in the respiratory rate. This increase was about 15% above the original value and persisted for about 3 hr., after which time the rate gradually fell.

The respiration of skin maintained in 2-deoxyglucose, 3-deoxyglucose and *N*-acetylglucosamine was similar to that of skin maintained without substrate; apparently they could not act as an energy source nor were they toxic. *N*-Acetylglucosamine, however, supported respiration for a period immediately after addition; this may perhaps be due to traces of acetate as an impurity which the skin could utilize (Cruickshank *et al.* 1957).

Further experiments with equimolar glucose and 1:5-anhydro-D-glucitol, 2-deoxyglucose, 3-deoxyglucose, *N*-acetylglucosamine and glucosone were carried out to see whether these substances were inert or whether they interfered with respiration on glucose. The results are included in Table 1, from which it can be seen that 3-deoxyglucose and *N*-acetylglucosamine apparently did not interfere with glycolysis. 2-Deoxyglucose markedly interfered with glucose utilization, respiratory survival being only 45% compared with 84% for skin maintained in glucose alone. Little respiratory suppression (relative to glucose media) was caused by 1:5-anhydro-D-glucitol and D-glucosone but interference with normal glucose metabolism may be inferred from the fact that although stimulation of respiration still occurred on mixed substrates it was less than that produced by 1:5-anhydro-D-glucitol and D-glucosone alone.

2-Deoxyglucose did not interfere with the normal metabolism of galactose.

Phosphate esters formed by skin

In initial experiments five extracts prepared from guinea-pig skin maintained on 0·02 M-glucose were chromatographed as described earlier. The radioautographs on development showed a number of spots; a tracing of one of these is shown in Fig. 2. A large dense spot always appeared (no. 5); this was shown to be inorganic phosphate. With inorganic phosphate as a reference spot, R_I values were recorded for the two directions of chromatography. The reproducibility of the pattern of these spots between different experiments was very good, though the identification of spots never depended upon this reproducibility. Fig. 3 shows a composite diagram constructed from the five chromatograms. The esters found were numbered arbitrarily. Twenty-two esters were found consistently on each chromatogram and a further six occurred on some chromatograms (these six are denoted by circles in Fig. 3).

Three experiments were carried out with human skin (surplus to grafting operations); the pattern and number of phosphate esters detected appeared to be identical with those found in guinea-pig skin. The positions of the majority of the esters from human skin were well within the limits shown in Fig. 3.

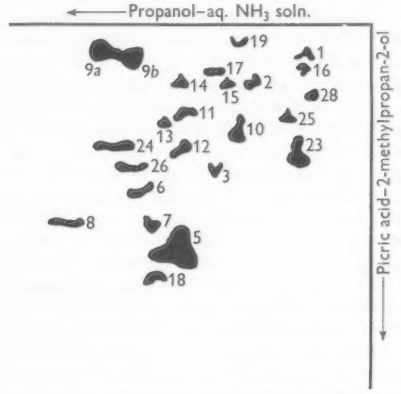


Fig. 2. Phosphate esters from guinea-pig skin maintained on a medium containing [^{32}P]orthophosphate and glucose. An extract of the skin was subjected to chromatography in two dimensions. The sketch illustrates the radioautograph of a typical chromatogram. See Table 2 for a key to the identified spots.

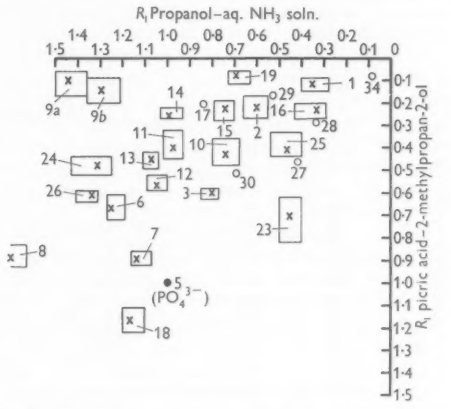


Fig. 3. Diagram showing the position on chromatograms of phosphate esters from mammalian skin. Crosses denote the means and rectangles the limits of R_I values in five experiments. Circles denote the position of esters not detected on every chromatogram. See Table 2 for a key to the identified spots.

Further experiments were made with guinea-pig skin extracts to identify as many of these phosphate esters as possible. This was done by mixing phosphate esters at 0·02M with the skin extract as markers. After chromatography the marker was detected by a spray reagent. The coincidence of the colour with the radioautograph image was taken as evidence that the marker substance was present in the skin extract. Usually the coloured spot was larger than the image owing to the relative sensitivity of the two methods; only for inorganic phosphate was the image larger than the blue spot. Distortions of the spot occasionally occurred, e.g. a crescent shape occurred with ATP, but these were accepted where there was reasonable symmetry of image and blue spot.

Usually two separate experiments were made with each marker. Altogether 14 of the regularly occurring skin phosphate esters were identified in this way; these are listed in Table 2 under the heading 'Skin maintained on glucose'. In addition to the compounds listed there was some reason to believe that spot no. 1 was uridine triphosphate, spot no. 2 was uridine monophosphate and spot no. 29 was guanosine triphosphate, but owing to impurities in the reference samples of nucleotides these identifications remain uncertain.

Experiments were made to study the effect of varying the substrate on these phosphate esters. A series of extracts were made from guinea-pig skin maintained on the following substrates at 0·02M: mannose, 1:5-anhydro-D-glucitol, 2-deoxyglucose, 3-deoxyglucose, glucosone, glucosamine, *N*-acetylglucosamine and also without substrate. The radioautographs from skin maintained without substrate, on *N*-acetylglucosamine and 3-deoxoglucose were each similar to those from glucose cultures except that the intensity of the spots was greatly reduced and some of the nucleotides were missing.

Skin maintained on mannose produced chromatograms indistinguishable from chromatograms of skin maintained on glucose.

Chromatograms from skin maintained on other substrates (1:5-anhydro-D-glucitol, 2-deoxyglucose, glucosone and glucosamine) each showed several phosphate esters not normally present (see Table 2, under the heading 'Skin maintained on various substrates'). With 2-deoxyglucose most of the 'normal' spots were eliminated. The intensity of the spots from skin maintained on 1:5-anhydro-D-glucitol, glucosone and glucosamine was reduced compared with those from glucose medium but it was greater than spots from systems without substrate.

Table 2. *Phosphate esters in mammalian skin cells*

Esters were separated by two-dimensional chromatography on paper and provisionally identified by superimposition of the radioautograph image of the [^{32}P]ester formed in the skin cells with the colour reaction of an authentic sample of the ester added to the skin extract. The table is based on 41 chromatograms and R_I values are listed correspondingly; for example, spot no. 20 showed R_1 1·25 in propanol–aq. NH_3 soln. (solvent 1) and 0·79 in picric acid–aq. 2-methylpropan-2-ol (solvent 2) on the same chromatogram. R_1 is the ratio of the distance moved by the phosphate ester to the distance moved by orthophosphate.

Spot no.	Ester	R_I Solvent 1	R_I Solvent 2
	Skin maintained on glucose		
5	Orthophosphate	1·00	1·00
6	Fructose 6-phosphate	1·19, 1·26	0·64, 0·68
7	3-Phospho-D-glyceric acid	1·05, 1·16	0·87, 0·88
9*b*	Uridine diphosphoglucose	1·29, 1·27	0·16, 0·19
10	Uridine diphosphate	0·77, 0·78	0·35, 0·40
11	Adenosine monophosphate	0·95, 1·01, 0·89	0·33, 0·41, 0·33
12	Mannose 6-phosphate	1·08	0·54
13	Glucose 6-phosphate	1·05, 1·09	0·46, 0·41
14	Adenosine diphosphate	0·95, 0·94, 0·89	0·22, 0·25, 0·21
16	Guanosine diphosphate	0·38	0·24
18	Phosphoenolpyruvic acid	1·13, 1·16	1·16, 1·09
19	Adenosine triphosphate	0·71, 0·66, 0·67	0·15, 0·12, 0·06
23	Fructose 1:6-diphosphate	0·40, 0·46	0·68, 0·78
24	Glucose 1-phosphate	1·28, 1·35, 1·33	0·51, 0·51, 0·41
25	Pyrophosphate	0·49, 0·52, 0·56	0·42, 0·45, 0·47
	Skin maintained on various substrates*		
20	2-Deoxyglucose 6-phosphate	1·25, 1·34	0·79, 0·77
21	Glucosone 6-phosphate	1·25, 1·36	0·57, 0·59
31	1:5-Anhydro-D-glucitol-6-phosphate	1·31, 1·34	0·77, 0·70
36	Glucosamine 6-phosphate	1·32, 1·38	0·54, 0·57

* The substrates were the unesterified sugar derivatives corresponding to the esters shown.

It was shown that in each case the 'new' esters were distinct from the 'normal' esters of the glucose medium by mixing each extract in turn with a glucose-medium extract and chromatographing the mixture. It was possible to identify one of the new esters from each substrate by mixing a solution of the C-6 phosphate of that substrate into the appropriate skin extract and following the procedure described earlier for ester identification. The R_I values of these compounds have been included in Table 2 under the heading 'Skin maintained on various substrates'.

DISCUSSION

Skin maintained on glucose for 24 hr. shows a 15% decrease in respiration rate after this time, whereas that of skin without substrate decreases by about 65%. These values correspond with those obtained by Cruickshank *et al.* (1957) from similar experiments. The phosphate esters detected in skin maintained on glucose included the majority of the glycolysis-cycle intermediates and certain nucleotides, notably derivatives of adenosine, guanosine and uridine. A similar pattern of esters was obtained from skin without substrate but the intensity of the radioautograph spots was much reduced and many of the nucleotides could not be demonstrated. This indicates that glycolysis was much reduced and the difference between the respiratory rates of skin maintained on glucose and maintained without substrate is consistent with this.

The finding that 3-deoxyglucose and *N*-acetylglucosamine neither act as substrates for skin nor interfere with its respiration on glucose suggested that the pattern of skin phosphate esters would also be unaffected. Chromatography showed that this was indeed the case. This observation is consistent with the inability of hexokinase to act upon *N*-acetylglucosamine (Grant & Long, 1952) and the failure of 3-deoxyglucose to interfere with glycolysis (Woodward, Cramer & Hudson, 1953).

In contrast, 1:5-anhydro-D-glucitol, glucosamine and glucosone act as energy sources for skin metabolism but these substances are not so effective as glucose (Table 1). The respiratory stimulation caused on addition of glucosone and 1:5-anhydro-D-glucitol is interesting; certain bacterial toxins produce a similar effect (Lawrence, 1959) but the cause is unknown. The three substrates, 1:5-anhydro-D-glucitol, glucosamine and glucosone, when mixed with glucose do not inhibit skin respiration as compared with that of cultures on glucose alone. The respiratory stimulation caused by 1:5-anhydro-D-glucitol and glucosone which still occurs in the presence of glucose, though to a less extent, implies an interference with skin metabolism though the effect cannot be precisely defined. The fact that 1:5-anhydro-D-glucitol and glucosone are phosphorylated by hexokinase suggests that there is an interference with glucose phosphorylation. Similarly, glucosamine 6-phosphate was found in extracts from skin maintained on a mixture of glucose and glucosamine. It is known that glucosone and glucosamine inhibit glucose breakdown by tumour cells (Yushok & Batt, 1957).

In skin maintained on glucosone and 1:5-anhydro-D-glucitol four new esters were found: two for each substrate (glucosone nos. 21 and 22; 1:5-anhydro-D-glucitol nos. 31 and 32). Esters nos. 21 and 31 were identified as glucosone 6-phosphate and 1:5-anhydro-D-glucitol 6-phosphate respectively. The intensity of radioactivity of these esters was not strong enough to suggest that they accumulate in skin. The identity of the other two is unknown but it is possible that no. 22 could be glucosone 1-phosphate formed by the action of phosphoglucomutase, which is known to convert glucosamine 6-phosphate into glucosamine 1-phosphate (Brown, 1953; Distler, Merrick & Roseman, 1958). Clearly 1:5-anhydro-D-glucitol 6-phosphate cannot be affected by phosphoglucomutase and in this case the second ester may be some further intermediate in its breakdown by skin. Chemically it is difficult to visualize how 1:5-anhydro-D-glucitol can be metabolized further than the 6-phosphate. Studies with ^{14}C might throw more light on this problem. The presence of these 6-phosphates is in agreement with previous work; it is known that all are phosphorylated by brain hexokinase in the presence of ATP (Harpur & Quastel, 1949; Sols & Crane, 1954).

Mannose is also known to be phosphorylated by hexokinase, and since chemically it is so closely related to glucose it is not surprising to find that skin can utilize it equally well. The presence of mannose 6-phosphate in normal skin maintained on glucose is noteworthy but certain polysaccharides of connective tissue such as keratosulphate are known to contain mannose (Meyer, Davidson, Linker & Hoffman, 1956), so the presence of its 6-phosphate is perhaps to be expected.

Respiration studies made with 2-deoxyglucose as substrate indicate that it does not affect skin but when mixed with glucose considerable inhibition of glucose metabolism occurs (Table 1). This is in agreement with observations made by Yushok & Batt (1957) and Woodward & Hudson (1954). Chromatography showed the presence of two phosphate esters (nos. 4 and 20) which do not normally occur in skin. These two esters showed a greater radioactivity than did the esters formed by glucosone, the esters of glucosamine and 1:5-anhydro-D-glucitol and the esters found normally, which suggests that they had accumulated in the skin

cells. No. 20 was shown to be 2-deoxy-D-glucose 6-phosphate, which is reported to accumulate in the cells of the rat diaphragm when these are cultured on 2-deoxyglucose (Kipnis & Cori, 1959). The identity of the second ester (no. 4) is unknown.

Galactose supported skin respiration to the same extent as glucose, although hexokinase does not act on galactose under normal conditions (Sols & Crane, 1954) and it seems likely that it is converted into galactose 1-phosphate by galactokinase, an enzyme previously detected in yeasts (Caputto, Leloir, Trucco, Cardini & Paladini, 1949). So far no chromatograms have been prepared from skin maintained on galactose to see whether this is likely, but an experiment in which skin was maintained on equimolar galactose and 2-deoxyglucose showed that 2-deoxyglucose had no effect on the respiration of skin under these conditions. This suggests that an enzyme other than hexokinase is responsible for the utilization of galactose by skin.

The combination of respiration measurements with an examination of phosphate esters in skin provides a useful biochemical approach. Further use of the method should provide information about the metabolism of other sugars by skin and may also have application in determining the site of action of toxic materials both synthetic and microbial in origin.

SUMMARY

1. The phosphate esters of skin have been examined in conjunction with measurements of respiratory activity.

2. Skin maintained on glucose regularly produced detectable amounts of 22 phosphate esters. From time to time six others were observed.

3. Fourteen of the regularly occurring compounds were identified chromatographically and the majority were intermediates in normal glycolysis or were nucleotides.

4. Respiration studies made with other substrates showed that galactose and mannose were as effective as glucose in supporting skin respiration, but *N*-acetylglucosamine, 2-deoxy-D-glucose and 3-deoxy-D-glucose did not support respiration. 1:5-Anhydro-D-glucitol, glucosone and glucosamine supported skin respiration to some extent but were not as efficient as glucose. When mixed with glucose the following inhibited normal glucose metabolism: 1:5-anhydro-D-glucitol, 2-deoxyglucose, glucosone and glucosamine.

5. Hexose 6-phosphates not normally present in skin were formed from 1:5-anhydro-D-glucitol, 2-deoxy-D-glucose, D-glucosone and glucosamine; of these 2-deoxy-D-glucose 6-phosphate appeared to accumulate in the skin cells. No new phosphate esters were produced from 3-deoxy-D-glucose or from *N*-acetylglucosamine.

REFERENCES

Brook, S. A. (1959). Ph.D. Thesis: University of London.
Brown, D. H. (1951). *Biochim. biophys. Acta*, **7**, 487.
Brown, D. H. (1953). *J. biol. Chem.* **204**, 877.
Caputto, R., Leloir, L. F., Trucco, R. E., Cardini, C. E. & Paladini, A. C. (1949). *J. biol. Chem.* **179**, 497.
Cruickshank, C. N. D. (1954). *Exp. Cell Res.* **7**, 374.
Cruickshank, C. N. D., Trotter, M. D. & Cooper, J. R. (1957). *Biochem. J.* **66**, 285.
Distler, J. J., Merrick, J. M. & Roseman, S. (1958). *J. biol. Chem.* **230**, 497.
Field, J. (1948). *Meth. Med. Res.* **1**, 299.
Foster, A. B. & Stacey, M. (1955). *J. chem. Soc.* p. 1778.
Grant, P. T. & Long, C. (1952). *Biochem. J.* **50**, xx.
Hanes, C. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.
Harpur, R. P. & Quastel, J. H. (1949). *Nature, Lond.*, **164**, 693.
Johnstone, J. M. & Mitchell, I. L. S. (1953). *Biochem. J.* **55**, xvii.
Kilbourne, E. D. (1959). *Nature, Lond.*, **183**, 271.
Kipnis, D. M. & Cori, C. F. (1959). *J. biol. Chem.* **234**, 171.
Lawrence, J. C. (1959). *Brit. J. exp. Path.* **40**, 8.
Leuthardt, F. & Testa, E. (1951). *Helv. chim. acta*, **34**, 931.
Meyer, K., Davidson, E., Linker, A. & Hoffman, P. (1956). *Biochim. biophys. Acta*, **21**, 506.
Ness, R. K., Fletcher, H. G. & Hudson, C. S. (1950). *J. Amer. chem. Soc.* **72**, 4547.
Overend, W. G., Stacey, M. & Staněk, J. (1949). *J. chem. Soc.* p. 2341.
Pierce, O. R. & Kane, T. G. (1954). *J. Amer. chem. Soc.* **76**, 301.
Sols, A. & Crane, R. K. (1954). *J. biol. Chem.* **210**, 581.
Wick, A. N., Drury, D. R., Nakada, H. J. & Wolfe, J. B. (1957). *J. biol. Chem.* **224**, 963.
Woodward, G. E., Cramer, F. B. & Hudson, M. T. (1953). *J. Franklin Inst.* **256**, 577.
Woodward, G. E. & Hudson, M. T. (1954). *Cancer Res.* **14**, 599.
Woodward, G. E. & Hudson, M. T. (1955). *J. Franklin Inst.* **259**, 543.
Yushok, W. D. & Batt, W. G. (1957). 131*st Meet. Amer. chem. Soc.* 12D.

Regulation of Glucose Uptake by Muscle

3. THE EFFECTS OF INSULIN, ANOXIA, SALICYLATE AND 2:4-DINITROPHENOL ON MEMBRANE TRANSPORT AND INTRACELLULAR PHOSPHORYLATION OF GLUCOSE IN THE ISOLATED RAT HEART*

BY H. E. MORGAN,† P. J. RANDLE AND D. M. REGEN
Department of Biochemistry, University of Cambridge, England, and Department of Physiology, Vanderbilt University, Nashville, Tennessee, U.S.A.

(*Received* 20 *May* 1959)

In previous papers (Randle & Smith, 1958*a*, *b*) evidence was presented that insulin, anoxia and substances such as salicylate or 2:4-dinitrophenol, which inhibit oxidative phosphorylation, increase the uptake of glucose and D-xylose by isolated rat diaphragm by accelerating the transfer of these sugars across the muscle-cell membrane. The conclusion was drawn that the transfer process for sugars in muscle is inhibited by a substance generated during oxidative phosphorylation and that insulin activates the transfer process by interfering with the action of this substance.

The present study was undertaken to examine the effects of anoxia, salicylate and 2:4-dinitrophenol on the uptake of sugars by muscle in more detail. For this purpose the uptake of a sugar such as glucose which is phosphorylated by muscle can be considered to involve three sequential steps: (i) passage of sugar from the interior of the capillary to the cell membrane, (ii) transfer of sugar across the cell membrane, and (iii) phosphorylation of sugar within the cell (with sugars such as D-xylose or L-arabinose, which are not phosphorylated, step iii is not involved). The rate of uptake of glucose depends on the resistance to the flow of the sugar through each of these steps individually but the one which has the greatest resistance will be predominantly rate-limiting. It follows therefore that any factor which accelerates uptake must affect this step in particular. One consequence of this may be that another step becomes rate-limiting.

The isolated perfused rat heart has been used for measurement of the uptake process in these investigations. Pertinent information obtained previously with this tissue is as follows. In the heart from the normal animal passage of sugars between the capillary and the cell membrane occurs very rapidly and does not affect the rate of uptake appreciably (Morgan, Cadenas & Park, 1958). Transfer across the cell membrane, on the other hand, appears to constitute a major barrier to the flow of glucose and is rate-limiting for uptake in the absence of insulin (Park, 1955; Morgan & Park, 1958). The kinetics of the transfer process, competition between sugars for transfer and the action of inhibitors such as phlorrhizin show that this transfer step involves transport of the sugar by combination with a specific site on the cell membrane (Bronk & Fisher, 1957; Park, Reinwein, Henderson, Cadenas & Morgan, 1959). In this connexion transport could be considered as the first reaction in glucose metabolism, preceding phosphorylation by the hexokinase system. When transport is accelerated by insulin (Levine & Goldstein, 1952) the capacity for phosphorylation may be exceeded, as shown by the accumulation of free glucose inside the cell (Park & Johnson, 1955; Fisher & Lindsay, 1956). Under these conditions phosphorylation becomes the rate-limiting step for glucose uptake.

With these considerations in mind the effects of anoxia, salicylate and 2:4-dinitrophenol on the transport and phosphorylation of sugars in the perfused isolated heart have been investigated and compared with those of insulin.

EXPERIMENTAL

Procedures

Perfusion medium. Heart perfusion was carried out with a bicarbonate-buffered salt solution (Krebs & Henseleit, 1932) containing D-[^{12}C]sorbitol (0·5 mg./ml.) and D-[$^{14}C_6$]-sorbitol (0·12 μc/ml.; 6·2 mc/m-mole; The Radiochemical Centre, Amersham, Bucks). The medium was maintained at pH 7·4 at 37° by gassing with $O_2 + CO_2$ (95:5). D-Glucose, D-xylose, D-galactose or L-arabinose (T. Kerfoot and Co. Ltd.) was added at the concentration given in the tables.

Animals. Hearts were obtained from male albino Wistar rats of 200–300 g. wt., fed on a stock laboratory diet (Bruce & Parkes, 1949). Animals had free access to water at all times but food was withheld for 18–20 hr. before each experiment. The rats were injected intraperitoneally with heparin (200 units; Evans Medical Supplies Ltd.) 15–60 min. before the experiment.

Perfusion apparatus. Heart perfusion was carried out in an apparatus described elsewhere (Morgan, Henderson & Park, in preparation).

* Part 2: Randle & Smith (1958*b*).

† Investigator of Howard Hughes Medical Institute.

Procedure. Rats were killed by decapitation and were bled. The heart was rapidly removed and dropped into ice-cold medium. It was then attached to the perfusion cannula, washed through with about 8 ml. of medium and then placed in the perfusion chamber and perfusion begun. In all experiments, except for arabinose wash-out, perfusion involved re-circulation of about 7 ml. of medium for 15 or 30 min. At the end of perfusion the heart was detached from the cannula, dropped into ice-cold medium and the chambers were opened to facilitate rapid chilling. It was then blotted on filter paper and frozen on a planchet resting on solid carbon dioxide and kept frozen until analyses were made.

L-*Arabinose wash-out.* The heart was first allowed to accumulate L-arabinose by perfusion for 15 min. under aerobic conditions with medium containing the pentose (15 mg./ml.). Arabinose-free medium was then passed through the heart for 5 min. (without re-circulation) to remove extracellular pentose. At this point the collecting vessel was changed. Perfusion with arabinose-free medium was then continued for a further 10 min. with collection of the perfusate from the heart. During this period of perfusion the collecting vessel was changed at intervals of 1–2 min. Pentose was estimated in the collections obtained during the latter (10 min.) period of perfusion and in the heart at the end of the perfusion.

Analytical methods

D-*Glucose*, D-*xylose*, L-*arabinose* or D-[$^{14}C_6$]*sorbitol.* Analyses of heart muscle for these substances were made on extracts prepared by boiling the muscle in 4 ml. of water for 15 min. (a procedure which extracts these substances completely). The extracts were deproteinized with $Zn(OH)_2$ (Somogyi, 1945). Samples of the perfusion medium were suitably diluted and deproteinized in the same way. Glucose was determined photometrically with glucose oxidase, peroxidase and *o*-dianisidine (see Randle & Smith, 1958*a*). Xylose was estimated photometrically by the *p*-bromoaniline method (Roe & Rice, 1948) and arabinose by the orcinol method (Mejbaum, 1939). When xylose was estimated in muscle extracts or samples of incubation medium containing glucose in addition to xylose, glucose was first removed by incubation with yeast. When arabinose was estimated by the orcinol method, muscle extracts were shaken with moist ion-exchange resins (Dowex 50, H^+ form and Duolite A-4, OH^- form) to decrease the blank. These resins did not remove any pentose from the extract. For assay of D-[$^{14}C_6$]sorbitol, samples were pipetted on to aluminium planchets containing a disk of lens tissue. After the addition of a drop of a saturated aqueous solution of polyvinyl alcohol, the samples were dried under an infrared lamp and counted beneath a thin end-window Geiger–Müller tube. At least 1000 counts were recorded for each sample. Since the weight of material added in the sample was less than 0·25 mg./cm.^{2}, no correction was applied to these estimates of radioactivity.

Total water was determined by weighing the muscle before and after freeze-drying.

Calculations

Glucose uptake. This was calculated as mg. of glucose disappearing from the perfusion medium/g. of wet muscle/hr. of perfusion.

Total muscle water, glucose, pentose or sorbitol spaces. These spaces, expressed as ml./100 g. of wet muscle, were calculated from formulae given by Randle & Smith (1958*b*).

Arabinose wash-out. The exit of arabinose from the muscle cells was calculated as the percentage of initial intracellular arabinose washed out by the perfusion medium per minute. The initial intracellular arabinose was calculated by summing the arabinose removed during perfusion and the residual arabinose in the heart at the end of perfusion. The rate of exit (μg. of arabinose/g. of wet heart/min.) was also calculated as a function of the intracellular concentration. The latter was calculated by dividing the pentose content of the heart at each time period by the intracellular fluid volume (total water minus sorbitol space).

Intracellular accumulations. To determine the intracellular accumulation of glucose or xylose, the glucose or xylose space was compared with the volume of extracellular fluid (determined by measuring the sorbitol space). Glucose or xylose was deemed to be present within the cell if the glucose or xylose space was greater than the sorbitol space.

RESULTS

Effect of anoxia and insulin, separately or in combination, on the uptake and accumulation of glucose

The effects of anoxia and insulin, separately or in combination, on the uptake and accumulation of glucose are shown in the upper part of Table 1. Under aerobic conditions insulin more than doubles uptake of glucose and causes accumulation of free glucose within the cardiac-muscle cells (i.e. the glucose space is greater than the volume of extracellular fluid estimated with sorbitol). Anoxia also more than doubles uptake of glucose but the sugar did not accumulate within the cells under these conditions. The combined influence of insulin and anoxia led to an even greater acceleration of glucose uptake and the level of uptake was approximately twice that attained with either insulin (under aerobic conditions) or anoxia and approximately four times that attained in the absence of insulin under aerobic conditions. Free glucose did not accumulate within muscle cells when insulin was present under anaerobic conditions, in clear contrast with the effect of the hormone under aerobic conditions.

With deprivation of oxygen the heart stopped beating within 2–3 min. The perfusion flow was, however, usually increased by about 50% and the pressure somewhat reduced. In appearance the heart was flaccid and somewhat swollen and in keeping with this the extracellular (sorbitol) space and total water content were increased (Table 2). Neither sorbitol space nor water content was affected by insulin.

Effects of salicylate and 2:4-dinitrophenol

The effects of salicylate (5 mM) and 2:4-dinitrophenol (0·25 mM) on glucose uptake are shown in Table 1. Salicylate caused a small but nevertheless

Table 1. *Effect of insulin, anoxia, sodium salicylate, 2:4-dinitrophenol, D-galactose and phlorrhizin on the uptake of glucose and the glucose and sorbitol spaces of the perfused rat heart*

The number of hearts perfused is given in parentheses. Initial concentration of glucose was 5·5 mM.

Experiment	Period of perfusion (min.)	Insulin (0·1 unit/ml.)	Glucose uptake (mean±S.E.M.) (mg. of glucose/g. of wet heart/hr.)	Space (mean±S.E.M.) (ml./100 g. of wet muscle)	
				Glucose	Sorbitol
Aerobic control	0–15	0	4·9±0·4 (11)	35·0±2·6	36·0±0·8
	0–30	0	4·3±0·2 (10)	29·7±2·1	35·6±1·0
Aerobic	0–15	+	10·0*±0·6 (8)	45·8*±1·7	36·6±1·0
Anaerobic	0–15	0	12·1*±0·6 (9)	37·3±1·8	41·2*±2·3
	0–30	0	9·5*±0·7 (8)	32·0±3·0	41·9*±0·8
Anaerobic	0–15	+	17·6*†±1·9 (8)	31·2†±1·3	44·0±1·0
Aerobic control	0–30	0	4·3±0·2 (10)	29·7±2·1	35·6±1·0
Aerobic control + sodium salicylate (5 mM)	0–30	0	6·9*±0·4 (8)	35·3±2·6	43·1*±1·4
Aerobic control + 2:4-dinitrophenol (0·25 mM)	0–15	0	6·2*±0·4 (8)	64·4*±2·5	30·8±1·4
	0–30	0	3·6±0·6 (8)	—	—
Anaerobic control	0–15	0	12·1±0·6 (9)	37·3±1·8	41·2±2·3
Anaerobic control + D-galactose (44 mM)	0–15	0	8·6*±0·6 (7)	38·0±1·7	47·0±1·8
Anaerobic control + phlorrhizin (3 mM)	0–15	0	2·7*±0·3 (7)	37·8±1·1	44·0±2·0

* $P < 0.01$ *vs.* appropriate control. † $P < 0.01$ *vs.* aerobic + insulin.

significant increase in glucose uptake. The glucose space was increased but nevertheless remained smaller than the sorbitol space. 2:4-Dinitrophenol also increased glucose uptake but only during the first 15 min. of perfusion. In the subsequent period, uptake fell below the control rate and a large quantity of free sugar was found inside the cell.

Salicylate increased the sorbitol space, whereas 2:4-dinitrophenol reduced it. These different effects of salicylate and 2:4-dinitrophenol may be related to their effects on the mechanical and physical state of the tissue. When perfused with medium containing salicylate about 50% of the hearts ceased to beat after 10–15 min.; the remainder continued beating for the full 30 min. but at a slow rate (80–120/min.). There was no obvious correlation between the contractile activity and the glucose uptake. Those hearts which ceased to beat with salicylate appeared flaccid and swollen; the perfusion flow and pressure were not altered. With perfusion of 2:4-dinitrophenol, on the other hand, contraction ceased in all cases after 2–3 min. The coronary flow was maintained only by virtue of a 20–30 mm. Hg rise in perfusion pressure. The heart appeared smaller in size and was apparently in a state of contracture.

Effect of D-galactose and phlorrhizin

It appeared important, for reasons to be discussed later, to determine whether D-galactose or phlorrhizin, which inhibit the uptake of glucose under aerobic conditions (Fisher & Lindsay, 1956; Morgan & Park, 1958), exert a similar effect under anaerobic conditions. The presence of D-galactose in a molecular ratio to glucose of 8:1 reduced uptake of glucose moderately (lower part of Table 1). Phlorrhizin at a molecular ratio to glucose of 0·55:1 inhibited uptake of glucose very markedly. These effects of galactose and phlorrhizin were not associated with accumulation of intracellular free glucose.

Reversibility of the effects of anoxia

It was found that the effects of anoxia on glucose uptake could be promptly and nearly completely reversed by restoration of aerobic conditions. In Expt. 3 of Table 2 the heart was first perfused for 15 min. anaerobically; the medium was sampled and a stimulated uptake found as noted previously (cf. Expt. 2). The incubation was then continued for a second 15 min. period under aerobic conditions. The glucose uptake was restored to the same value as that observed in hearts incubated aerobically from the beginning (Expt. 1). The sorbitol space and water content were also restored to typically aerobic values, but the glucose space remained somewhat elevated. The contractile function of the heart was regained very promptly under oxygen. The muscle began to beat again within 30 sec. and within 5 min. regained a normal rate of 200–240 beats/min.

Substrate-dependence with insulin and anoxia

Expt. 4 of Table 2 shows that the extremely high rate of glucose uptake during 15 min. of perfusion under anaerobic conditions was maintained during a second 15 min. period of perfusion. On the other hand, if the heart had first been incubated anaerogically for 15 min. without glucose then uptake of glucose was impaired during a second 15 min. period of incubation under anaerobic conditions with medium containing the sugar. The sorbitol space was considerably reduced under these conditions and substantial amounts of glucose accumulated within the cell.

Effects of anoxia and insulin on the efflux of L-*arabinose*

The effect of anoxia on transport alone was studied with L-arabinose. This pentose crosses the cell membrane by the same mechanism as glucose (Morgan & Park, 1958), but unlike glucose it is not phosphorylated. It was convenient to measure the efflux of arabinose from the intracellular fluid of hearts which had accumulated a large amount of the sugar during a preliminary perfusion (see Experimental section). As discussed elsewhere (Park *et al.* 1959), efflux rates provide a more sensitive and reliable measure of transport than

Table 2. *Reversibility of effects of anoxia on glucose uptake and glucose, sorbitol and total water spaces of the perfused rat heart: effect of substrate deprivation and anaerobiosis* (15 *min.*) *on the subsequent metabolism of glucose*

Each vertical column shows a separate experimental series with the first 15 min. of perfusion in the upper part and the second 15 min. in the lower part. The number of hearts is given in parenthesis. Initial concentration of glucose in the perfusion medium was 5·5 mM.

Expt. no.	1	2	3	4	5
0–15 min.	Aerobic control	Anaerobic control	Anaerobic then aerobic	Anaerobic + insulin	Anaerobic + insulin without glucose
Gas phase	O_2+CO_2	N_2+CO_2	N_2+CO_2	N_2+CO_2	N_2+CO_2
Glucose uptake (mean ± S.E.M.; mg./g./hr.)	—	12·1±0·6 (9)	11·5±1·2 (6)	17·6±1·9 (8)	No substrate
Space (mean ± S.E.M.; ml./100 g.)					
Glucose	—	37·3±1·8	—	31·2±1·3	—
Sorbitol	—	41·2±2·3	—	44·0±1·0	—
Total water	—	81·5±0·3	—	81·5±0·3	—
15–30 min.					
Gas phase	O_2+CO_2	—	O_2+CO_2	N_2+CO_2	N_2+CO_2
Glucose uptake (mean ± S.E.M.; mg./g./hr.)	4·6±0·8 (7)	—	4·7*±0·3 (6)	16·9±1·5 (6)	7·0†±0·8 (8)
Space (mean ± S.E.M.; ml./100 g.)					
Glucose	29·7±2·1	—	39·2±1·9	32·6±2·5	49·6†±2·6
Sorbitol	35·6±1·1	—	36·9*±2·0	42·7±0·8	33·7†±2·4
Total water	79·1±0·3	—	80·2*±0·4	81·2±0·5	81·0±0·4

* $P > 0.05$ *vs.* Expt. 1, lower panel. † $P < 0.01$ *vs.* Expt. 4, lower panel.

Table 3. *Effect of anoxia ± insulin on the efflux of* L-*arabinose from the perfused rat heart*

The number of hearts perfused is given in parenthesis. Aerobic *vs.* anaerobic (no insulin) $P < 0.01$ for all times; aerobic (no insulin) *vs.* aerobic (insulin) $P < 0.01$ for all times; aerobic *vs.* anaerobic (both with insulin) $P > 0.1$; anaerobic (no insulin) *vs.* anaerobic (insulin) $P < 0.01$ for all times.

Cumulative time of wash-out (min.)	Percentage of intracellular arabinose removed (mean ± S.E.M.)			
	No insulin		Insulin (0·1 unit/ml.)	
	Aerobic (5)	Anaerobic (5)	Aerobic (4)	Anaerobic (7)
1	9·6±0·8	13·2±1·1	31·5±2·0	29·7±1·1
2	18·5±1·7	26·0±1·7	50·8±2·4	48·0±1·6
3	26·7±2·6	37·2±2·2	63·1±2·3	59·4±2·0
4	33·7±3·0	46·5±2·4	70·6±2·2	67·0±2·2
6	45·0±3·3	62·0±2·8	78·8±2·2	75·4±2·2
8	53·7±3·0	73·0±3·5	83·1±2·1	80·7±1·8
10	60·2±2·6	82·7±3·5	85·6±2·0	84·4±1·6

Table 4. *Effect of glucose* (30 mM) *and phlorrhizin* (3 mM) *on the accumulation of* D-*xylose in the perfused rat heart under aerobic or anaerobic conditions*

The period of perfusion was 10 min. The number of hearts perfused is given in parentheses. Initial concentration of D-xylose in the perfusion medium was 10 mM.

Expt.	Space (mean ± S.E.M.) (ml./100 g.) Total water	Sorbitol	Xylose	Intracellular xylose† as percentage of extracellular xylose concn. (mean ± S.E.M.)
Aerobic: no glucose (6)	79·2±0·3	36·5±1·0	62·8±0·8	62·2±1·9
Aerobic: with glucose (5)	79·6±0·3	35·2±1·4	55·0*±1·1	44·2*±2·1
Anaerobic: no glucose (6)	81·5±0·3	42·9±1·0	67·9±1·8	65·6±4·8
Anaerobic: with glucose (10)	83·3±0·2	45·9±1·3	55·1*±1·1	24·6*±2·9
Anaerobic: no glucose with phlorrhizin (4)	83·5±1·0	45·3±1·7	44·0*±1·9	0*

* $P < 0{\cdot}01$ *vs.* appropriate control.

† Calc. from $\frac{\text{xylose space} - \text{sorbitol space}}{\text{total water} - \text{sorbitol space}} \times 100$.

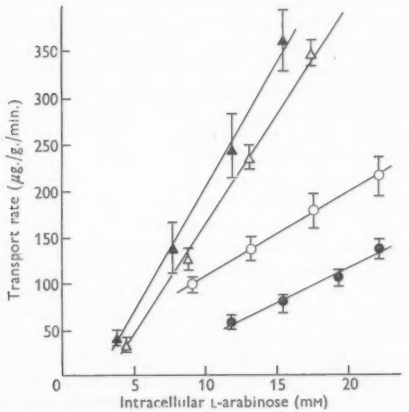


Fig. 1. Effect of anoxia on the outward transport of L-arabinose from the perfused rat heart, with and without insulin. The concentration of insulin was 0·1 unit/ml. The number of hearts are given in Table 3. Mean values are plotted, together with twice the S.E. ●, Aerobic (no insulin); ▲, aerobic (with insulin); ○, anaerobic (no insulin); △, anaerobic (with insulin).

the determination of rates of intracellular accumulation.

As shown in Table 3, anoxia caused a rise in the rate of efflux of intracellular arabinose. This effect was similar to that of insulin but of smaller magnitude. In the heart already treated with insulin, anoxia caused no further acceleration.

Since transport rates are a function of sugar concentration a more valid comparison is obtained when the efflux data are recalculated to give transport rates as a function of the intracellular pentose concentration, as shown in Fig. 1. It can be seen that transport was a linear function of concentration under all the conditions tested. By comparison with the control, anoxia caused an approximately twofold acceleration and insulin or insulin + anoxia about a fourfold acceleration.

Effect of D-*glucose and phlorrhizin on accumulation of* D-*xylose*

As shown in Table 4, D-xylose entered the cell rapidly under aerobic conditions and, after about 10 min., reached a mean concentration inside the cell which was about 60% of that in the perfusate. Comparison of these results with those reported elsewhere (Morgan *et al.* 1959) indicates that this approximates to the maximal intracellular pentose concentration. Presumably for this reason no significant increase in accumulation could be shown with anoxia, although transport of xylose was presumably accelerated as is transport of glucose and arabinose. The addition of glucose to the medium significantly depressed accumulation of xylose both aerobically and anaerobically. Phlorrhizin was a very potent inhibitor of xylose accumulation and completely prevented any measurable penetration of the cell by xylose under anaerobic conditions.

DISCUSSION

This investigation has confirmed the earlier conclusion of Randle & Smith (1958*a*, *b*) that anoxia, like insulin, increases the rate of transport of glucose across the muscle-cell membrane. On the other hand, these studies have revealed an additional effect of anoxia on the rate of phosphorylation of glucose by cardiac muscle which is not shown by insulin and which could not be detected in earlier studies with isolated diaphragm by Randle & Smith.

In interpreting our data transport of glucose is considered to be rate-limiting for glucose uptake if no free sugar accumulated intracellularly and phosphorylation of glucose is considered to be rate-limiting if free glucose was detected in the muscle

cell. Furthermore since sugars may be transported across the cell membrane in both directions (Morgan *et al.* 1958) the rate of net inward transport of glucose (inward − outward transport measured as glucose uptake) will only approximate to the true rate of inward transport if transport is the rate-limiting step in glucose uptake (i.e. if no free glucose accumulates within the cells).

In hearts perfused under aerobic conditions without addition of insulin (aerobic control) transport was the rate-limiting step in glucose uptake. Anoxia increased glucose uptake (2·5-fold over aerobic control) and transport remained rate-limiting. We conclude from this that anoxia accelerates transport of the sugar. When insulin was present under aerobic conditions glucose uptake was increased (twofold over control) but phosphorylation of glucose now became rate-limiting. On the other hand, when insulin was present under anaerobic conditions glucose uptake was increased still further (to 3·6-fold over aerobic control) but now transport and not phosphorylation was the rate-limiting step. Thus anoxia prevented phosphorylation from becoming rate-limiting in the presence of insulin and we conclude therefore that anoxia must stimulate phosphorylation of glucose in addition to accelerating transport of the sugar. In cardiac muscle, then, changes in the rate of membrane transport and in the potential rate of phosphorylation of glucose are of importance in the Pasteur effect. Moreover, these observations demonstrate that insulin is still effective under anaerobic conditions (cf. Randle & Smith, 1958*a*).

These interpretations are supported and strengthened by data obtained from measurements of the outward transport of L-arabinose. The transport of this pentose may be used as a convenient indicator of glucose transport because arabinose is not phosphorylated and studies of competitive inhibition show that both sugars are transported by the same system (Morgan & Park, 1958). Furthermore, the transport process for L-arabinose is freely reversible and subject to hormonal influences in either direction (Morgan *et al.* 1958). Anoxia approximately doubled the rate of arabinose transport whereas insulin or insulin + anoxia effected about a fourfold increase. The changes in the rate of arabinose transport induced by anoxia or by insulin + anoxia are thus comparable to the changes in the rate of glucose transport which they effect (as measured by glucose uptake). On the other hand, the effect of insulin on the rate of arabinose transport under aerobic conditions is much greater than its effect on glucose uptake. This is perhaps not surprising since free glucose accumulates in muscle cells under these conditions and outward transport of the sugar may be taking place to a significant extent. It is to be expected therefore that the effect of insulin on the rate of glucose transport under aerobic conditions will be greater than its effect on net inward transport of the sugar (see above). It seems reasonable therefore to conclude that anoxia effects about a twofold acceleration of glucose or arabinose transport and insulin about a fourfold increase. These changes are very similar to the changes in glucose uptake of isolated diaphragm induced by these factors (Randle & Smith, 1958*a*). On the other hand, a fourfold increase in glucose uptake of isolated diaphragm was seen with insulin under both aerobic and anaerobic conditions. In this tissue free glucose does not accumulate in cells when insulin is present under aerobic conditions (Randle & Smith, 1958*b*).

Although both insulin and anoxia accelerate the transport of glucose and L-arabinose in cardiac muscle the effects of the two factors acting together are not additive. This would suggest that they affect the same transport process and at some common point (cf. Randle & Smith, 1957; 1958*a*).

Our studies also show that when the heart is incubated anaerobically without glucose the tissue shows an impaired capacity to utilize glucose on subsequent incubation with the sugar. Dickens & Greville (1933) first showed, with brain slices, that glucolysis may become impaired when a tissue is incubated under anaerobic conditions without glucose. Elliott & Rosenfeld (1958) have made a similar observation with brain slices and they attribute this phenomenon to depletion of adenosine triphosphate, which is necessary for phosphorylation of glucose. An analogous phenomenon has also been observed in erythrocytes stored for long periods in the absence of metabolizable substrate. These cells fail to utilize glucose until primed by the addition of a nucleoside such as adenosine, which can apparently result in synthesis of adenosine triphosphate by a mechanism which does not involve the initial expenditure of a high-energy phosphate bond (Prankerd, 1956). Our observations with cardiac muscle are essentially similar and they may also be attributable to impaired phosphorylation of glucose owing to depletion of adenosine triphosphate. This is supported by our finding that free glucose accumulates within the cell under these conditions.

Sodium salicylate and 2:4-dinitrophenol both appear to increase membrane transport of sugar in a manner similar to anaerobiosis (Randle & Smith, 1958*b*). The transport rate of hearts perfused with medium containing sodium salicylate is accelerated, as shown by the increased glucose uptake. 2:4-Dinitrophenol appears initially to have the same effect but in addition must have an inhibitory effect on phosphorylation of glucose because glucose uptake subsequently declines and free sugar accumulates within the cell.

The question can be raised whether acceleration of the entry of sugars into the cell with anaerobiosis is due simply to a non-specific breakdown of membrane integrity or to a more specific effect on the sugar-transport system. The present work appears to show that anoxia exerts a specific effect on the transport system. Thus if the effect was due to breakdown of the cell membrane it is to be expected that sorbitol would enter the cell as well as the sugars studied, and the evidence suggests that sorbitol did not enter the cell under these conditions. However, the sorbitol space did increase in the presence of salicylate and under anaerobic conditions in the presence of glucose. We believe that this was due to changes in the volume of extracellular fluid, and not to penetration of the cell by sorbitol, for the following reasons. The sortibol space did not increase progressively when the time of anaerobic incubation was prolonged from 15 to 30 min. Furthermore, restoration of aerobic conditions after anaerobic incubation led to a prompt reduction of the sorbitol space to normal and this was associated with an equivalent loss of tissue water. Moreover, the sorbitol space was actually reduced when 2:4-dinitrophenol was present or when hearts were incubated under anaerobic conditions without glucose. Further points of evidence accrue from measurements of arabinose and xylose transfer. The efflux of arabinose from muscle was increased by anoxia in the absence but not in the presence of insulin. If anoxia was accelerating transfer of arabinose by causing breakdown of the cell membrane then we would have expected it to accelerate regardless of whether the hormone was present or not. Finally D-galactose and phlorrhizin hindered glucose entry and glucose and phlorrhizin inhibited xylose entry under anaerobic conditions. The preservation of these inhibitory effects under anaerobic conditions indicates that these sugars were still entering by a specific transport process and not by a process of free diffusion.

SUMMARY

1. The mechanism by which anoxia, salicylate and 2:4-dinitrophenol stimulate glucose uptake in the perfused isolated rat heart has been investigated. The effect of these agents has been compared with that of insulin.

2. Anoxia increases glucose uptake by accelerating transport of glucose across the cell membrane and also by increasing intracellular phosphorylation of glucose. In the absence of insulin the effect of anoxia on transport is of chief importance since this is the predominantly rate-limiting step for glucose uptake. In the presence of insulin, the effect on phosphorylation is the more important because the hormone alone accelerates transport sufficiently to make phosphorylation of glucose the rate-limiting step.

3. Anoxia induces a twofold increase in glucose transport whereas insulin causes a three- to four-fold increase. The effects of these agents on transport and phosphorylation stimulate glucose uptake to the following extent: anoxia, 2·5-fold; insulin, twofold; anoxia + insulin, 3·6-fold.

4. Salicylate (5 mM) and 2:4-dinitrophenol (0·25 mM) also stimulate the transport of glucose across the cell membrane. 2:4-Dinitrophenol also inhibits intracellular phosphorylation of glucose.

5. The effects of anoxia, salicylate and 2:4-dinitrophenol appear to involve acceleration of the specific monosaccharide-transport process in the membrane and the effects are not due to a breach of membrane integrity.

6. These observations offer a partial explanation for the Pasteur effect in muscle and support the earlier suggestion of Randle & Smith (1957, 1958*a*, *b*) that membrane transport of glucose in muscle is regulated in some way by the availability of high-energy phosphate.

We wish to thank Professor F. G. Young and Professor C. R. Park for making it possible for us to collaborate in this investigation and for their interest and encouragement. The work was supported by grants from the Medical Research Council, the American Heart Association, the U.S.P. Health Service and the National Science Foundation.

REFERENCES

Bronk, M. S. & Fisher, R. B. (1957). *J. Physiol.* **136**, 435.
Bruce, H. M. & Parkes, A. S. (1949). *J. Hyg., Camb.*, **47**, 201.
Dickens, F. & Greville, G. D. (1933). *Biochem. J.* **27**, 1134.
Elliott, K. A. C. & Rosenfeld, M. (1958). *Canad. J. Biochem. Physiol.* **36**, 721.
Fisher, R. B. & Lindsay, D. B. (1956). *J. Physiol.* **131**, 526.
Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyl. Z.* **210**, 33.
Levine, R. & Goldstein, M. S. (1952). *Brookhaven Symp. Biol.* **5**, 73.
Mejbaum, W. (1939). *Hoppe-Seyl. Z.* **258–9**, 117.
Morgan, H. E., Cadenas, E. & Park, C. R. (1958). *Abstr. Comm. 4th int. Congr. Biochem., Vienna*, p. 104.
Morgan, H. E. & Park, C. R. (1958). *Fed. Proc.* **17**, 1099.
Park, C. R. (1955). In *The Hypophyseal Growth Hormone, Nature and Actions*, p. 394. Ed. by Smith, R. W., jun., Gaebler, O. H. & Long, C. N. H. New York. McGraw-Hill Book Co. Inc.
Park, C. R. & Johnson, L. H. (1955). *Amer. J. Physiol.* **182**, 17.
Park, C. R., Reinwein, D., Henderson, M. J., Cadenas, E. & Morgan, H. E. (1959). *Amer. J. Med.* **26**, 674.
Prankerd, T. A. J. (1956). *Biochem. J.* **64**, 209.
Randle, P. J. & Smith, G. H. (1957). *Biochim. biophys. Acta*, **25**, 442.
Randle, P. J. & Smith, G. H. (1958*a*). *Biochem. J.* **70**, 490.
Randle, P. J. & Smith, G. H. (1958*b*). *Biochem. J.* **70**, 501.
Roe, J. H. & Rice, E. W. (1948). *J. biol. Chem.* **173**, 507.
Somogyi, M. (1945). *J. biol. Chem.* **160**, 69.

Purification of the Vitamin B_{12}-Active Material from the Haemoflagellate, *Crithidia fasciculata*

By F. SANDERS and G. R. SEAMAN
Carter Physiology Laboratory, University of Texas Medical Branch, Galveston, Texas, U.S.A.

(*Received* 4 *December* 1958)

Extracts of the haemoflagellate, *Crithidia fasciculata*, replace vitamin B_{12} for the growth of *Escherichia coli* 113–3, for the soil bacterium referred to as 'Lochhead 38' and for *Euglena gracilis* (Seaman & Sanders, 1957). The substance differs, however, from members of the B_{12} series of vitamins in that its activity is completely destroyed by exposure for 24 hr. at room temperature to pH 3·5 or 10·7 (Seaman & Sanders, 1957). The B_{12} vitamins are stable at pH 3·5 and at pH 10·7 undergo only slight degradation, with the release of unidentified fragments which replace cyanocobalamin in the growth of *E. coli* 113–3; alkaline destruction of the *Crithidia* material does not release such fragments. In spite of these differences of stability in acid and alkali, the biological activity of the material from *Crithidia* suggests that it is a functional member of the B_{12} series of vitamins.

The present report describes a procedure for the partial purification of the vitamin B_{12}-like material from *Crithidia* and describes some of its biological properties. The active material will be referred to as vitamin B_{12}(Cr). It is not suggested that this be adopted as a term for a newly described member of the vitamin B_{12} series, but rather that the term is used merely for convenience of reference.

EXPERIMENTAL

Materials used and assay procedures

Crithidia fasciculata. Stock cultures were grown in tubes containing the yeast hydrolysate–liver concentrate–haemin medium described by Cowperthwaite, Weber, Packer & Hutner (1953). Cultures were grown at 25°, and transfers were made at monthly intervals. Large-scale cultures were grown for 4–5 days at 25° in stainless-steel drums (capacity 200 l.,) containing 50 l. of the Cowperthwaite 'final' synthetic medium (Cowperthwaite *et al.* 1953) to which sterile glucose and vitamins were added after autoclaving. Each drum was inoculated with 1 l. of a 4- to 5-day-old culture which had been grown in the same synthetic medium. Aeration was provided by drawing air through the drums by means of a vacuum line.

To obtain information about the physiological importance of vitamin B_{12}(Cr), the effect of the purified vitamin on the growth of *Crithidia* was studied. Assays were carried out in 25 ml. nephelometer flasks containing 4 ml. of the 'final' synthetic medium of Cowperthwaite *et al.* (1953); sorbitol (1·5%, w/v) was used as energy source. The flasks were covered with aluminium caps. Both flasks and caps were obtained from the Bellco Glass Co., Vineland, New Jersey, U.S.A. Flasks were inoculated with one drop of a 1:20 dilution of the stock culture, the diluent being the synthetic medium. The cultures were incubated at 25° and growth was periodically measured in a Klett–Summerson photometer equipped with a green filter (Klett–Summerson no. 65).

Vitamin B_{12} *assays.* These were carried out by the filter-paper disk method with *E. coli* 113–3, details of which have been described (Sanders & Seaman, 1959). Purified samples of vitamin B_{12}(Cr) were assayed with the chrysomonad, *Ochromonas malhamensis*, which is specific in its requirement for cyanocobalamin (Ford & Hutner, 1955). Stock cultures of this organism were maintained on the basal medium described by Hutner, Bach & Ross (1956), supplemented with 0·1 μmg. of cyanocobalamin/ml. Assays were carried out in 25 ml. nephelometer flasks containing 5 ml. of basal medium and covered with aluminium caps. One drop of a 1:20 dilution of the stock culture was used as inoculum, the diluent being unsupplemented basal medium. Flasks were illuminated with a 40 w fluorescent lamp (Ken-Rad, F40, T-12, type CW) at a distance of 20 cm. and incubated for 5 days at 25°. Growth was measured in a Klett–Summerson photometer with a green filter (Klett–Summerson no. 65).

Extraction and purification of vitamin B_{12}(Cr)

Preliminary extraction. Haemoflagellates were collected by passage through a Sharples supercentrifuge at 4°. The organisms were washed by centrifuging twice with 0·1 M-phosphate buffer, pH 8·5, once with 0·9% NaCl and once with water. They were then suspended in 0·03 N-HCl to give a concentration of approx. 80 mg. of solids/ml. After the addition of 0·5 mg. of NaCN/ml., hydrolysis was carried out in a boiling-water bath for 45 min. After cooling to 4° the pH was adjusted to 4·5–5·0 with 2 N-KOH and the insoluble material was removed by centrifuging.

Purification of extract. Ethanol (95%, v/v; 5 vol.) at 4° was added slowly to the extract with stirring, and the mixture was allowed to stand at 4° for 4 hr. The precipitate was removed by centrifuging and the ethanolic extract was evaporated to near dryness *in vacuo*. The residue was dissolved in a minimum volume of 3 mN-HCl and was extracted into 5 vol. of water-saturated phenol. The phenol-rich layer was extracted three times with equal volumes of water and the protein-free aqueous solutions were combined and evaporated to dryness *in vacuo*. The residue was dissolved in the minimum volume of 0·05 M-phosphate buffer, pH 6·0. The redissolved extract was taken up in a small amount of ether-washed Celite 535 (Johns-Manville Co. Ltd.) and was placed on top of a 3 in. × 30 in. chromatographic column packed with Celite 535 (Swim & Krampitz, 1953). Several

Table 1. *Summary of purification of vitamin* B_{12}(Cr)

Vitamin activity was assayed by the filter-paper-disk method with *E. coli* 113–3.

Fraction	Total dry wt. of solids (mg.)	Total volume (ml.)	Total vitamin B_{12} activity (μmg.)	Activity per mg. dry wt. of solids (μmg.)
Cell extract	184800	2400	720	0·004
Phosphate extract	3720	620	625	0·17
Celite eluate	11	25	250	22·7

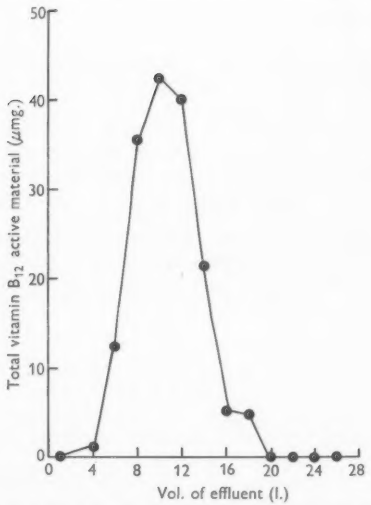


Fig. 1. Elution of vitamin B_{12}(Cr) from chromatographic column packed with Celite 535. The solvent system was the organic phase of a mixture of butan-2-ol–0·05 M-phosphate buffer (pH 6·0)–4% (w/v) KCN (100:50:0·25, by vol.). Vitamin B_{12} activity was assayed by the filter-paper-disk method with *E. coli* 113–3.

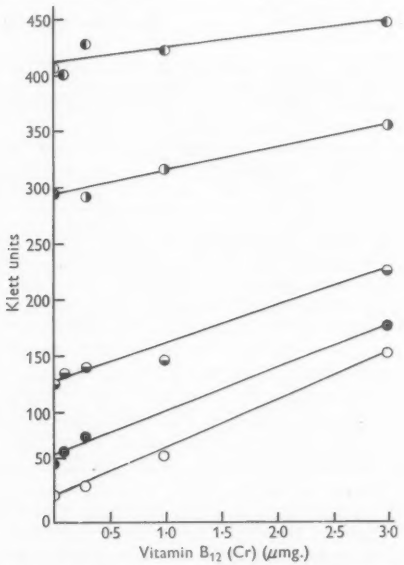


Fig. 2. Growth response of *Ochromonas malhamensis* to vitamin B_{12}(Cr) and to combinations of vitamin B_{12}(Cr) with cyanocobalamin. The amounts of added cyanocobalamin and vitamin B_{12}(Cr) were determined by assay with *E. coli* 113–3. ○, No added cyanocobalamin; ●, 0·01 μmg. of cyanocobalamin; ◒, 0·03 μmg. of cyanocobalamin; ◑, 0·1 μmg. of cyanocobalamin; ◐, 1·0 μmg. of cyanocobalamin.

litres of the organic phase of butan-2-ol–0·05 M-phosphate buffer (pH 6·0)–4% (w/v) KCN (100:50:0·25, by vol.) were passed through the column under a pressure sufficient to deliver 2 l. of eluate/hr., and collections were made of successive 1 l. volumes. Fig. 1 shows that the maximum vitamin B_{12}(Cr) activity was eluted between 8 and 12 l. These five fractions of tawny-coloured solution were combined and evaporated *in vacuo* to a volume of 25 ml. The concentrate was a reddish brown colour. The degree of purification achieved by the procedure is shown in Table 1.

RESULTS

Activity of vitamin B_{12}(Cr) *for* Ochromonas. Fig. 2 shows that purified vitamin B_{12}(Cr) replaces cyanocobalamin for the growth of *Ochromonas*; however, it is only one-hundredth as active, on the basis of cyanocobalamin equivalents for growth of *E. coli* 113–3. When added in the presence of cyanocobalamin, vitamin B_{12}(Cr) augments the growth response. As illustrated in the figure, augmentation is maximal at low levels of cyanocobalamin. As the concentration of cyanocobalamin is increased the stimulation by vitamin B_{12}(Cr) decreases, until at levels of cyanocobalamin which support half-maximal to maximal growth, addition of vitamin B_{12}(Cr) is without appreciable effect.

Effect of vitamin B_{12}(Cr) *on growth of* Crithidia. Fig. 3*A* shows that addition of vitamin B_{12}(Cr) to cultures of *Crithidia* shortened the lag period and increased the total growth. Although the shortening of the lag phase of growth is not proportional to the amount of vitamin B_{12}(Cr) added, Fig. 3*B*

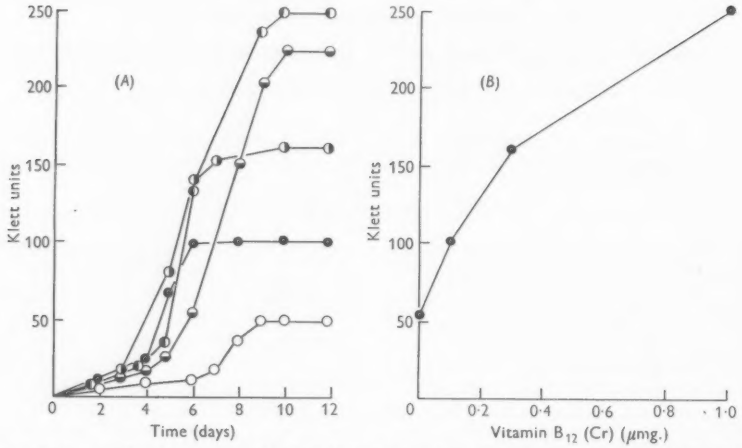


Fig. 3. Effect of vitamin B_{12}(Cr) on growth of *Crithidia fasciculata*. The amounts of vitamin B_{12}(Cr) added were determined as vitamin B_{12} equivalents by assay with *E. coli* 113-3 by the filter-paper-disk method. (*A*) Time course of growth response. ○, No added vitamin; ●, 0·1 μmg. of vitamin B_{12}(Cr); ◑, 0·3 μmg. of vitamin B_{12}(Cr); ◒, 1·0 μmg. of vitamin B_{12}(Cr); ◐, 3·0 μmg. of vitamin B_{12}(Cr). (*B*) Effect of vitamin B_{12}(Cr) on total growth (12 days' incubation) achieved by *Crithidia*. Data were taken from (*A*).

shows that the growth finally attained is dependent upon the concentration of added vitamin. Addition of either cyanocobalamin or factor B (the compound formed by removal of the nucleotide portion of cyanocobalamin) had no effect on the growth response.

It appears that vitamin B_{12}(Cr) is a growth factor for *Crithidia* and that the growing organisms are capable of synthesizing it in limited amounts. The growth of this organism is not stimulated by cyanocobalamin or by factor B. The vitamin is apparently also not identical with biopterin (2-amino-4-hydroxy-6-(1:2-dihydroxypropyl)-pteridine), the pteridine which under certain conditions is required for the growth of *Crithidia* (Patterson, Broquist, Albrecht, von Saltza & Stokstad, 1955) since in media containing large amounts of folic acid, as does the medium used in the experiments shown in Fig. 3, the pteridine requirement is abolished.

SUMMARY

1. The vitamin B_{12} activity [vitamin B_{12}(Cr)] from the haemoflagellate, *Crithidia fasciculata*, has been purified 5500-fold with a recovery of 34% of the initial activity present in cell extracts.

2. Vitamin B_{12}(Cr) is approximately one-hundredth as active as cyanocobalamin for the growth of *Ochromonas malhamensis*.

3. The growth of *Crithidia* is stimulated by vitamin B_{12}(Cr); the degree of stimulation is dependent upon the concentration of vitamin added.

This investigation was aided by a grant (E-159) from the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service, and from the Elsa U. Pardee Foundation.

REFERENCES

Cowperthwaite, J., Weber, M. M., Packer, K. & Hutner, S. H. (1953). *Ann. N.Y. Acad. Sci.* **56**, 972.
Ford, J. E. & Hutner, S. H. (1955). *Vitam. & Horm.* **13**, 101.
Hutner, S. H., Bach, M. K. & Ross, G. I. H. (1956). *J. Protozool.* **3**, 101.
Patterson, E. L., Broquist, H. P., Albrecht, A. M., Saltza, M. H. von & Stokstad, E. L. R. (1955). *J. Amer chem. Soc.* **77**, 3167.
Sanders, F. & Seaman, G. R. (1959). *J. Bact.* (in the Press).
Seaman, G. R. & Sanders, F. (1957). *Science*, **125**, 398.
Swim, H. E. & Krampitz, L. O. (1953). *J. Bact.* **67**, 418.

Effect of 1:1:1-Trichloro-2:2-di-(*p*-chlorophenyl)ethane (DDT) and Related Compounds on the Adenosine Triphosphate–Phosphate Exchange Catalysed by a Particulate Fraction from the Mosquito

By O. GONDA, A. KALUSZYNER and Y. AVI-DOR
Israeli Institute for Biological Research, Ness-Ziona, Israel

(*Received* 19 *March* 1959)

Many attempts have been made to relate the toxic action of 1:1:1-trichloro-2:2-di-(*p*-chlorophenyl)-ethane (DDT) to an interference with specific enzymes. DDT at a relatively high concentration (mM) was reported to inhibit cytochrome oxidase from both mammals and flies (Sacktor, 1949; Johnston, 1951; Morrison & Brown, 1954). A much lower concentration of DDT (50 μM) was found to suppress oxidative phosphorylation in a subcellular fraction from the adult housefly (Sacklin, Terrier & Remmert, 1955) and from the mosquito (Gonda, Traub & Avi-Dor, 1957). The incorporation of ^{32}P into ATP catalysed by insect respiratory particles proved also to be sensitive to DDT (Avi-Dor & Gonda, 1959). In work described in this paper an attempt was made to relate the inhibition of the adenosine triphosphate ^{32}P exchange by various DDT analogues to their solubility in the respiratory particles and to their toxicity for insect larvae.

EXPERIMENTAL

Materials. DDT and its analogues were either purified commercial products or synthesized. DDT (I), m.p. 108°, commercial product; 1:1-dichloro-2:2-di-(*p*-chlorophenyl)-ethane (II), m.p. 109–110° (Mosher, Cannon, Conroy, Van Strien & Spalding, 1946); 1:1:1-trichloro-2:2-di-(*p*-methoxyphenyl)-ethane (III), m.p. 88°, commercial product; 1:1-dichloro-2:2-di-(*p*-chlorophenyl)ethylene (IV), m.p. 88–89°, prepared by dehydrochlorination of DDT by ethanolic KOH (Grumitt, Buck & Jenkins, 1945); di-(*p*-chlorophenyl)-acetic acid (V), m.p. 164–166° (Grumitt, Buck & Egan, 1955); 1:1:1:2-tetrachloro-2:2-di-(*p*-chlorophenyl)ethane (VI), m.p. 92–93° (Grumitt, Buck & Jenkins, 1945); 1:1-di-(*p*-chlorophenyl)ethanol (VII), m.p. 69–70° (Grumitt, Buck & Becker, 1945); 1:1:1-trichloro-2:2-diphenylethane (VIII), m.p. 64–65° (Baeyer, 1872); 1:1-di-(*p*-chlorophenyl)-2:2:2-trifluoroethanol (IX) (Kaluszyner, Reuter & Bergmann, 1955); 1:1-di-(*p*-chlorophenyl)-2:2:2-trichloroethyl acetate (X), m.p. 128–129° (Bergmann & Kaluszyner, 1958). The other chemicals used, the composition of the buffers and the colony of *Aëdes aegypti* L. employed were as described by Avi-Dor & Gonda (1959).

Methods. The procedure for the isolation of respiratory particles from mosquito, the method of assay of the exchange between adenosine triphosphate (ATP) and ^{32}P catalysed by the respiratory particles and other analytical methods used have been described by Avi-Dor & Gonda (1959). The composition of the assay medium, unless indicated otherwise, was as follows: 2-amino-2-hydroxymethyl-propan-1:3-diol (tris) buffer, pH 7·4 (10 mM), sodium phosphate buffer, pH 7·4 (2·7 mM), 0·2 ml. of a stock solution of ^{32}P which contained approx. 1·5 μc/ml. (giving $1{\cdot}5 \times 10^5$ counts/min./ml. in a thin mica-window Geiger tube), sucrose (0·25 M), ATP (7 mM), human serum albumin (1%, w/v), ethylenediaminetetra-acetic acid (EDTA; 1 mM), respiratory particles from mosquito (equivalent to approx. 4 mg. dry wt./ml. of assay medium). The mixture was made up to 1·5 ml. with water. Time of incubation was 10 min.; temp. 28°. DDT and its analogues were added in propanol solution. The final concentration of propanol in the reaction mixture was 1% (w/v). The same amount of propanol was added to the controls.

For the determination of the median lethal dose (LD_{50}) of DDT analogues, 20 larvae of *Aëdes aegypti* L. (fourth-instar) were exposed to varying dilutions of the respective compound. The assay was carried out in Petri dishes of 9 cm. diameter at 28°; time of incubation was 24 hr.

For the determination of the partition of the DDT analogues between the aqueous phase of the reaction medium and between the suspended respiratory particles, the respiratory particles were equilibrated for 10 min. at 28° with the assay medium to which a known amount of the analogue in propanol solution has been added. Then the particles were separated by centrifuging at 8000 *g* for 7 min. at 0°, drained, transferred with the aid of 2 ml. of ethanol (96%, v/v) to a 10 ml. beaker and dried under an infrared lamp. The dried precipitate was dissolved by gentle warming in 3 ml. of a mixture of fuming nitric acid (1 vol.) and conc. sulphuric acid (1 vol.). The assay of the DDT analogues was performed by the method of Schechter, Soloway, Hayes & Haller (1945), in a sample of the solution so obtained. In many experiments the amount of DDT left in the supernatant was also determined. The practically complete recovery indicated that no metabolism of the compounds tested took place during the time of the experiment.

RESULTS

Factors affecting the partition of DDT and its analogues between the aqueous phase and the suspended respiratory particles

Effect of the concentration of serum albumin in the assay medium. The ATP–^{32}P exchange catalysed by insect particles is dependent on the addition of serum albumin to the assay medium (Avi-Dor & Gonda, 1959). Albumin had also a marked effect on

the stability of the DDT suspensions. When a reaction mixture containing 0·1 μmole of DDT/ml. was exposed to the centrifugal force (8000 *g* for 7 min.) which was used to separate the suspended respiratory particles, more than 0·09 μmole of DDT/ml. sedimented in the absence of serum albumin. This sedimentation was avoided only when the concentration of the albumin was at least 1 % (w/v) (Fig. 1). Serum albumin also reduced the amount of DDT which was absorbed by the respiratory particles and, as a corollary, the extent of inhibition of the exchange reaction (Fig. 2). In our experiments, the standard assay medium contained 1 % (w/v) of serum albumin. This level of albumin sufficed for maintaining the exchange reaction, for avoiding sedimentation of DDT and its analogues during centrifuging and yet allowed the absorption of these compounds by the particles.

Effect of the concentration of DDT, its analogues and of respiratory particles. Externally added substances with specific affinities toward lipids are accumulated by subcellular particles of high lipid content. This applies also to DDT and its analogues, which have hydrophobic character and high lipid solubilities. In Fig. 3 the amount of DDT and its analogues absorbed by a constant amount of particles is plotted against the concentration of these compounds in the medium. [A 0·1 mM-solution of DDT or of its analogues is completely transparent in the presence of 1 % (w/v) serum albumin. Since, however, it is not known whether these compounds form true solutions under such condition, 'concentration' denotes the amount of the compound (in μmoles) added in a propanol solution per l. of medium.] The partition of DDT and related compounds between the medium and the respiratory particles varied within wide limits. From a 0·1 mM-solution, 80 % of DDT, but only 5 % of 2:2-di-(*p*-chlorophenyl)acetic acid (V), was removed by the particles. Furthermore, the accumulation of DDT and its analogues depended on the ratio of the total amount of the compounds to the total amount of the respiratory particles in the

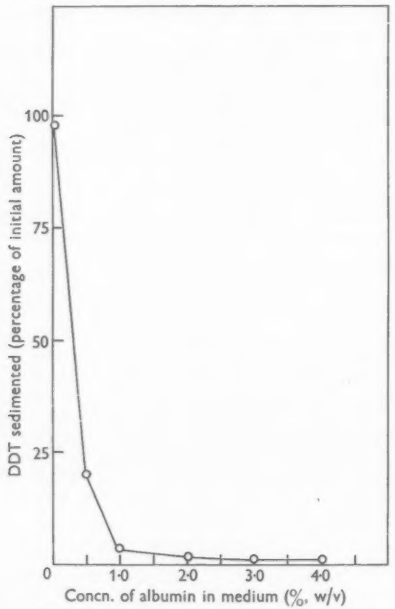


Fig. 1. Effect of the concentration of serum albumin on the sedimentation of DDT. From the standard assay medium ATP and ^{32}P were omitted, and the concentration of the serum albumin was varied as indicated. DDT was added (0·1 μmole/ml. of assay medium); total volume 15 ml. After centrifuging at 8000 *g* for 7 min. at 0° the amount of DDT sedimented was determined.

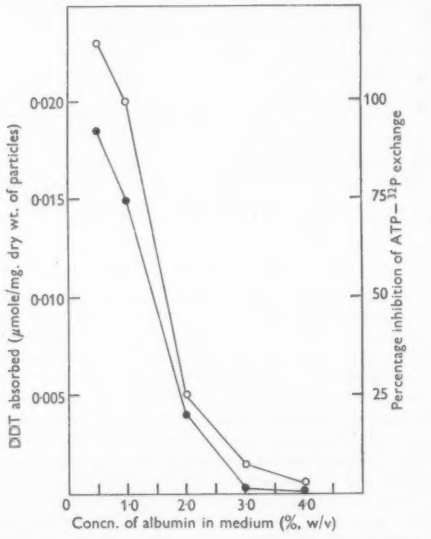


Fig. 2. Effect of the concentration of serum albumin on the reabsorption of DDT by the respiratory particles and on the extent of inhibition of the ATP–^{32}P exchange. ○, μmole of DDT absorbed/mg. dry wt. of particles; composition of the reaction mixture was as given in Fig. 1. ●, Extent of inhibition of ATP–^{32}P exchange; standard reaction mixture (see Experimental section). Respiratory particles were added in both experiments in a concentration equivalent to 4 mg. dry wt./ml. of medium. The amount of DDT added was 0·1 μmole/ml. of assay medium. A correction was applied for the amount of the DDT which sedimented under conditions of the assay in the absence of respiratory particles.

assay medium. This can be seen from Figs. 4 and 5. In Fig. 4, the variable factor is the volume of the reaction mixture while the concentration of DDT and the total amount of respiratory particles in the system are kept constant. In Fig. 5, the absorption of the DDT analogues is presented as a function of the total amount of the particles.

Inhibition of the adenosine triphosphate ^{32}P *exchange by DDT and related compounds*

In Table 1 the inhibitory action of various analogues of DDT on the exchange between ATP and inorganic phosphate on the one hand and of their toxicity for larvae of *Aëdes aegypti* L. on the other hand are compared. The concentration of the compounds that produced 50 % inhibition was read off from titration curves as shown in Fig. 6. The corresponding values for the concentration of the analogues in the respiratory particles proper was obtained from experiments described in Fig. 3. It can be seen from the Table that the concentration of various compounds in the particles, which produced a 50 % reduction in the rate of ATP–^{32}P exchange, varied within fairly narrow limits (0·01–0·017 μmole/mg. dry wt.). The corresponding variations of the concentration in the aqueous phase were ninefold and of the LD_{50} for mosquito larvae more than 2000-fold.

DISCUSSION

Theories based either on physical and chemical properties of DDT (electromagnetic and steric configuration, lipid solubility, dehydrohalogenation etc.) or on its interaction with specific enzymes failed to explain satisfactorily its physiological activity (cf. Wigglesworth, 1955). Tomizawa (1957) observed that when radioactive phosphate was injected into the abdomen of insects which were treated with DDT, the greatest changes in the labelling of the phosphorylated intermediates occurred in the ATP + ADP fraction. However, it was not shown whether this action is confined only to highly toxic derivatives of DDT.

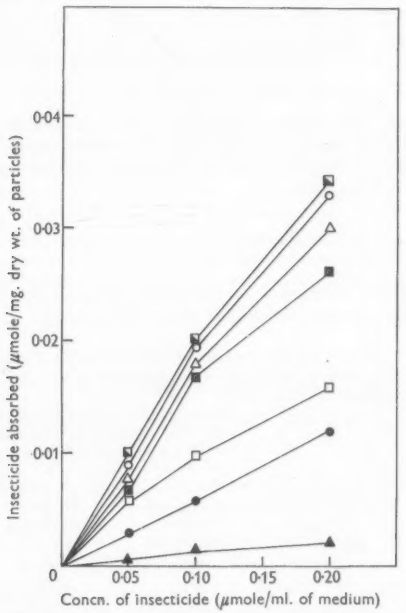


Fig. 3. Effect of the concentration of the insecticide in the aqueous phase on the amount absorbed by the particles. Composition of the assay medium and the conditions of the experiment were as given in Fig. 2. DDT analogues (see Table 1): ○, I; △, II; □, III; ●, IV; ▲, V; ■, VI; ◪, X.

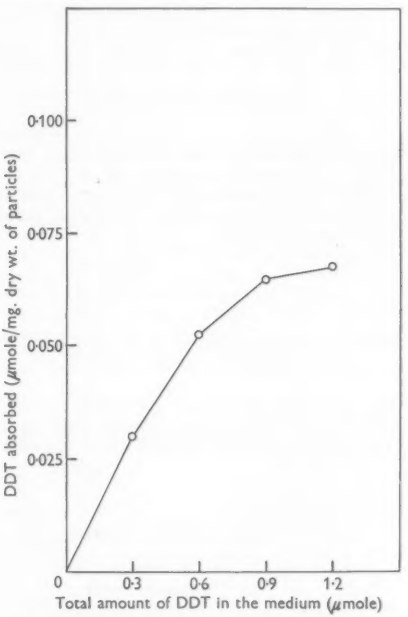


Fig. 4. Effect of variations in the total amount of DDT in the medium on the amount absorbed by the respiratory particles. Concentration of DDT in the medium was 0·1 mM. Respiratory particles were added in an amount corresponding to 4 mg./dry wt. The total amount of DDT in the system was changed by varying the volume of the medium. In other respects conditions of the reaction were as given in Fig. 1.

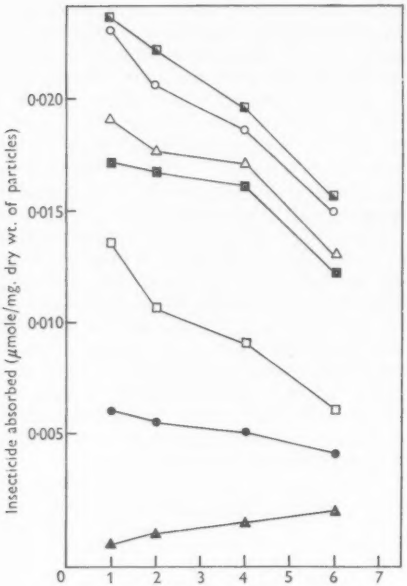


Fig. 5. Effect of the concentration of the respiratory particles on the amount of insecticide absorbed. Concentration of DDT and its analogues was 0·1 mM; serum albumin was 1% (w/v); other components were as in Fig. 1. Symbols for the analogues are as given in Fig. 3. Total volume was 15 ml.

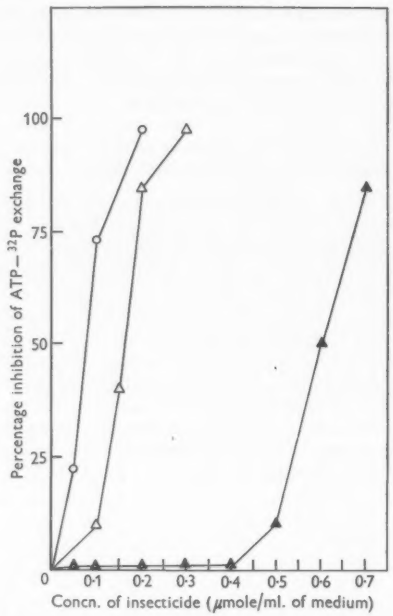


Fig. 6. Effect of the concentration of DDT and its analogues on the extent of inhibition of ATP–^{32}P exchange. Standard reaction medium was used. DDT analogues: ○, I; △, II; ▲, V.

Table 1. *Correlation between the toxicity of DDT analogues, their partition between the aqueous phase and the respiratory particles and their inhibitory effect on adenosine triphosphate–^{32}P exchange*

LD_{50} values for the various insecticides towards mosquito larvae were assayed as described under Methods. Concentration of respiratory particles was 4 mg. dry wt./ml. The amount of insecticides accumulated by the particles and the ATP–^{32}P exchange was measured as described under Methods.

DDT analogues tested	LD_{50} (μmole/ml.)	Concn. of DDT analogues when ATP–^{32}P exchange is inhibited by approx. 50%: In aqueous phase (μmole/ml.)	Concn. of DDT analogues when ATP–^{32}P exchange is inhibited by approx. 50%: In respiratory particles (μmole/mg. dry wt.)
1:1:1-Trichloro-2:2-di-(*p*-chlorophenyl)ethane (I)	0·0002	0·07	0·013
1:1-Dichloro-2:2-di-(*p*-chlorophenyl)ethane (II)	0·0005	0·10	0·017
1:1:1-Trichloro-2:2-(*p*-methoxyphenyl)ethane (III)	0·0005	0·15	0·010
1:1-Dichloro-2:2-di-(*p*-chlorophenyl)ethylene (IV)	>0·25	0·17	0·012
Di-(*p*-chlorophenyl)acetic acid (V)	>0·5	0·60	0·014
1:1:1:2-Tetrachloro-2:2-di-(*p*-chlorophenyl)ethane (VI)	0·03	0·07	0·012
1:1-Di-(*p*-chlorophenyl)ethanol (VII)	0·03	0·18	0·013
1:1:1-Trichloro-2:2-diphenylethane (VIII)	>0·25	0·07	0·012
1:1-Di-(*p*-chlorophenyl)-2:2:2-trifluoroethanol (IX)	0·03	0·05	0·015
1:1-Di-(*p*-chlorophenyl)-2:2:2-trichloroethyl acetate (X)	>0·2	0·07	0·013

In the present study the sensitivity of the ATP–^{32}P exchange reaction to DDT and to some related compounds has been compared in systems *in vitro*. A close correspondence has been revealed between the extent of inhibition of ATP–^{32}P exchange produced by various analogues of DDT and their concentation in the respiratory particles proper. This finding recalls the action of narcotic substances. According to Meyer (1937), the latter exert their physiological effect as a result of their preferred solubilities in the lipids of the cell. It is likely that the pronounced sensitivity of the ATP–^{32}P exchange reaction and that of the oxidative phosphorylation to DDT and its analogues is merely an expression of the fact that the phosphorylating electron-transport particles are rich in lipids. Various analogues of DDT which have similar partition characteristics, like compounds (I), (II), (III), (VIII) and (X), differ widely in their toxicity toward insect larvae. Compound (VIII) and compound (X) are approximately 1000 times less toxic than DDT. Thus the effect *in vivo* reflects not only the variation in lipid solubilities. One has to conclude either that the effect of DDT on the phosphorylative processes has no physiological significance at all or alternatively that, *in vivo*, unknown factors impede selectively the access of some of the analogues to the respiratory particles.

SUMMARY

1. The effect of 1:1:1-trichloro-2:2-di-(*p*-chlorophenyl)ethane (DDT) and its analogues on the adenosine triphosphate–^{32}P exchange reaction in mosquito sarcosomes has been compared.

2. All analogues of DDT tested inhibited the exchange reaction by nearly the same extent when their concentration in the particles reached the same level.

3. No correlation was found between the toxicity for mosquito larvae of the compounds tested and their effect *in vitro* on the exchange reaction.

We are indebted to Dr Rachel Galun for the cultivation of the test organisms, and to Mr S. Cohen for valuable help in the synthesis of the DDT analogues.

REFERENCES

Avi-Dor, Y. & Gonda, O. (1959). *Biochem. J.* **72**, 8.
Baeyer, A. (1872). *Ber. dtsch. chem. Ges.* **5**, 1094.
Bergmann, E. D. & Kaluszyner, A. (1958). *J. org. Chem.* **23**, 1306.
Gonda, O., Traub, A. & Avi-Dor, Y. (1957). *Biochem. J.* **67**, 487.
Grumitt, O., Buck, A. C. & Becker, E. I. (1945). *J. Amer. chem. Soc.* **67**, 2265.
Grumitt, O., Buck, A. C. & Egan, R. (1955). *Org. Synth.* (*Coll.*), **3**, 270.
Grumitt, O., Buck, A. C. & Jenkins, A. (1954). *J. Amer. chem. Soc.* **67**, 155.
Johnston, C. D. (1951). *Arch. Biochem. Biophys.* **31**, 375.
Kaluszyner, A., Reuter, S. & Bergmann, E. D. (1955). *J. Amer. chem. Soc.* **77**, 4165.
Meyer, K. H. (1937). *Trans. Faraday Soc.* **32**, 1062.
Morrison, P. E. & Brown, A. W. A. (1954). *J. econ. Ent.* **47**, 723.
Mosher, H. S., Cannon, M. R., Conroy, E. A., Van Strien, R. E. & Spalding, D. P. (1946). *Industr. Engng Chem.* **38**, 916.
Sacklin, J. A., Terrier, L. C. & Remmert (1955). *Science*, **122**, 337.
Sacktor, B. (1949). Ph.D. Thesis: Rutgers University, New Brunswick, N.Y.
Schechter, M. S., Soloway, S. B., Hayes, R. A. & Haller, H. L. (1945). *Industr. Engng Chem.* (*Anal.*) **17**, 704.
Tomizawa, Ch. (1957). In *Japanese Contributions to the Study of the Insecticide-Resistance Problem.* Kyoto, Japan: Institute of Insect Control, Kyoto University.
Wigglesworth, V. B. (1955). In *DDT*, vol. 1, p. 93. Ed. by Mueller, P. Basel: Birkhaeuser Verlag.

Synthesis of Glucosylfructoses by the Action of a Yeast α-Glucosidase

By G. AVIGAD
Laboratory of Microbiological Chemistry, Department of Biochemistry, The Hebrew University-Hadassah Medical School, Jerusalem, Israel

(*Received* 20 *April* 1959)

It has been reported (Herzog, 1913; Isaiev, 1926) that hydrolysis of maltose by yeast maltase is slightly inhibited by D-fructose. Similarly Avigad (1958) in a study of a hybrid yeast α-glucosidase has shown that D-fructose partially inhibited liberation of glucose from suitable α-glucoside substrates (sucrose, methyl α-glucoside or maltose).

It was of interest to inquire whether this inhibitory effect might be due to an ability of D-fructose to combine with the glucon-binding site of the enzyme, as suggested by Gottschalk (1950), or to an ability of fructose to compete with water as an acceptor for transferred α-glucosyl residues. Experiments described below show that the latter

is the case. The structure of several oligosaccharides formed by transglucosylation to carbinol positions in fructose in the presence of a yeast α-glucosidase is described.

MATERIALS AND METHODS

Oligosaccharides. All the materials used were chromatographically pure compounds. They had the physical constants and the phenylosazones as listed in Table 1. Isomaltose and isomaltotriose were isolated from a partial acid hydrolysate of *Leuconostoc mesenteroides* B-512 native dextran (Commercial Solvents Corp., Terre Haute, Ind., U.S.A.) as described by Turvey & Whelan (1957). Maltotriose was isolated from a salivary amylase digest of potato starch according to the procedures of Peat, Whelan & Kroll (1956).

Isomaltulose, maltulose, isomaltotriulose and maltotriulose were prepared by alkaline treatment of corresponding aldose sugars (Hough, Jones & Richards, 1953) as follows: 5 ml. of aq. conc. NH_3 soln. was added to 20 ml. of a 5% (w/v) solution of the aldose. After 24 hr. at 37° the solution was evaporated under reduced pressure at $< 30°$. The resulting syrup was dissolved in 10 ml. of water, 2 g. of $BaCO_3$ and 0·5 ml. of bromine were added and the mixture was agitated for 6 hr. at room temperature. After removal of bromine by aeration the solution was neutralized by addition of about 2 ml. of 0·2M-$Ba(OH)_2$. Ethanol (3 vol.) was added and the mixture was left overnight at 4°. The precipitate was removed by filtration and washed with 50 ml. of aq. 70% (v/v) ethanol. The filtrate was passed through an Amberlite MB1 mixed-bed resin and concentrated under reduced pressure. The concentrate was resolved quantitatively by chromatography on sheets of Whatman no. 3 MM filter paper, and bands of paper containing the desired ketose sugar were eluted with water. The yield of the ketose oligosaccharide was 25–29%.

Leucrose (Stodola, Sharpe & Koepsell, 1956) and 1-β-D-glucosylfructose (Pacsu, Wilson & Graf, 1939) were samples kindly donated by Dr F. H. Stodola and Professor E. Pacsu respectively. Uniformly labelled [^{14}C]fructose was obtained from The Radiochemical Centre, Amersham, Bucks.

Other sugars were commercial A.R. preparations.

Yeasts. Haploid-yeast strain 12836 of genotype MA MG su mz (Hestrin & Lindegren, 1952) was kindly provided by Professor C. C. Lindegren. This strain and the *Torulopsis glabrata* cells were grown with mild aeration. Medium and conditions of growth were as described by Hestrin & Lindegren (1950). The harvested washed haploid cells were dried in a stream of air at 35°.

Enzymes. The α-glucosidase was extracted from the dried yeast cells by incubating them for 3 hr. in 0·05M-$NaHCO_3$ soln. at 37°. This enzyme solution was found to be free from β-fructofuranosidase activity (Hestrin & Lindegren, 1952; Avigad, 1958).

The yeast invertase used was a concentrate supplied by British Drug Houses Ltd.

Chromatographic methods. Whatman no. 1 filter paper was used. The solvent systems were: I, butanol–ethanol–water (5:2:2, by vol.); II, butanol–pyridine–water (5:3:2, by vol.). The developing time was 72 hr. in descending and 8 hr. in circular chromatography.

The spray reagents used to obtain typically coloured spots with different sugars were: *p*-anisidine–HCl (Hough, Jones & Wadman, 1950) and aniline–diphenylamine–phosphoric acid (Schwimmer & Bevenue, 1956), as general sprays for revealing reducing sugars and for β-fructofuranoside in non-reducing sugars; urea–phosphoric acid (Wise, Dimler, Davis & Rist, 1955) for the detection of ketoses; alkaline aq. triphenyltetrazolium chloride (Wallenfels, 1950) for the detection of free reducing groups (cf. Feingold, Avigad & Hestrin, 1956).

Paper electrophoresis was conducted on Whatman no. 3 MM filter paper in 0·1M-sodium borate buffer, pH 10·0, at 20 v/cm., 25 mA during about 4 hr.

Quantitative determinations. Total hexose was determined by the anthrone reagent (Yemm & Willis, 1954); reducing sugar was determined cuprimetrically (Nelson, 1944), fructose with resorcinol (Roe, Epstein & Goldstein, 1949) and formaldehyde by the chromotropic acid method (O'Dea & Gibbons, 1953). Aldose was determined by a modification of the iodometric method of Macleod & Robison (1929) for use on a micro-scale.

RESULTS

Inhibitory effect of fructose on hydrolysis of α-glucosides

D-Fructose added to a reaction mixture containing α-glucosidase and a suitable α-glucoside as substrate caused a decrease in the rate at which free glucose was liberated (Table 2). The inhibition effected by fructose was of a non-competitive type (Fig. 1). Chromatographic analysis showed in such reaction mixtures the presence of a reducing ketose-containing product slightly lagging in mobility in relation to sucrose. In a paper chromatogram this product gave a spot which coalesced with that of sucrose and therefore it was difficult to distinguish it in samples containing sucrose as the initial substrate. With [^{14}C]fructose as inhibitor of hydrolysis, analyses of a radioautograph showed the product (tentatively regarded as 'glucosylfructose') to be the sole [^{14}C]oligosaccharide that was formed, except for a trace of radioactive product sometimes found at the level of a trisaccharide. No [^{14}C]-sucrose was found. Quantitative determinations clearly showed (Table 2) that the deficit in the liberation of glucose when fructose is present is nearly fully compensated by an amount of glucose transferred to fructose with formation of 'glucosylfructose'.

A small amount of the 'glucosylfructose' is also formed in systems in which sucrose was the only sugar added. This can be ascribed to liberation of fructose in the course of hydrolysis. Prolonged incubation of the enzymic reaction mixtures brings about the hydrolysis of both the initial substrates and the transfer products formed. Trace amounts of transfer products other than the 'glucosylfructose' were also detected in these reaction systems. The properties of these products and their assigned identities are given in Table 1.

Table 1. *Physical constants of formed oligosaccharides and reference compounds*

Reference sugars	$R_{glucose}$ Solvent I	$R_{glucose}$ Solvent II	$M_{glucose}$*	$[\alpha]_D^{22}$† (water)	Phenylosazone m.p.	Phenylosazone $[\alpha]_D^{22}$ (pyridine–ethanol; 2:3, by vol.)†	Comparative literature values
Sucrose	0·78	0·71	0·20	+67°	—	—	—
Maltose	0·64	0·45	0·36	+130	204–206°	+81°	Bates (1942); Peat, Roberts & Whelan (1952)
Isomaltose	0·54	0·41	0·66	+122	174–176	+42	Jeanes, Wilham, Jones, Tsuchiya & Rist (1953); Thompson & Wolfrom (1954)
Turanose	0·84	0·74	0·73	+75	202–204	+33	Peat *et al.* (1952); Pazur & Budovich (1956)
Leucrose	0·68	0·50	0·67	−8	186–188	—	Stodola, Sharpe & Koepsell (1956)
Maltulose	0·71	0·58	0·63	+57	202–204	+78	Peat *et al.* (1952); Hough, Jones & Richards (1953); Corbett & Kenner (1954); Yasumura (1954); White, Eddy, Petty & Hoban (1958)
Isomaltulose	0·68	0·53	0·59‡	+95	173–175	+44	Weidenhagen & Lorenz (1957)
1-β-Glucosylfructose	0·66	0·48	0·68‡	−59	—	—	Pacsu, Wilson & Graf (1939)
Isomaltotriose	0·26	0·18	0·81	+144	—	—	Jeanes *et al.* (1953); Wolfrom & Thompson (1957)
Maltotriose	0·32	0·23	0·32	+156	—	—	Peat, Whelan & Kroll (1956)
Isomaltotriulose	0·37	0·26	0·31	+120	—	—	—
Maltotriulose	0·42	0·34	0·35	+80	—	—	—
Products of transglucosylation:							Suggested identity:
a	0·64	0·48	0·70	+49§	—	—	1-α-Glucosylfructose
b	0·68	0·52	0·60	+94	174–176	+40	Isomaltulose
c	0·72	0·58	0·62	+60	201–203	—	Maltulose
d	0·54	0·40	0·67	+120	172–173	+42	Isomaltose
e	0·35	0·25	0·31	+118	—	—	Isomaltotriulose
f	0·26	0·18	0·80	+146	—	—	Isomaltotriose

* Index of migration in paper ionophoresis (Foster, 1953).
† Concentrations were between 0·7 and 1·0%.
‡ Comparative values for 6-β- and 1-β-fructosylfructose were 0·59 and 0·70 respectively (cf. Feingold, Avigad & Hestrin, 1956).
§ Yasumura (1954) described an unidentified glucosylfructose (AII) which may be identical with compound *a*.

Table 2. *Transfer of glucosyl from α-glucoside to fructose by an α-glucosidase of MA MG su mz yeast grown on different sugars*

Reaction mixture (0·2 ml.) consisted of 0·06 M-sodium phosphate buffer, pH 6·8, 0·2 M-α-glucoside as substrate and 0·1 ml. of enzyme. In a parallel series, 0·2 M-[^{14}C]-fructose (0·12 μc/μmole) was included. After incubation for 60 min. at 37°, reaction mixtures were inactivated by heating for 2 min. at 96°. Samples were removed, for determination of increase in aldose content by the iodometric method. Samples (10 μl.) from systems containing [^{14}C] sugars were applied to sheets of Whatman no. 3 MM filter paper for chromatographic separation. Radioautographs (6 days' contact with an X-ray-sensitive film) enabled the location of areas corresponding in mobility to a disaccharide sugar ($R_{sucrose}$ 0·86 in solvent 1) identified as glucosylfructose. These areas were cut from the chromatograms and extracted with 2 ml. of hot water. The eluents were dried and their radioactivity was measured in a Geiger–Müller counter with a thin end-window. Values indicate μmoles of product/ml. of reaction mixture.

		Enzyme from cells grown on methyl-α-glucoside			Enzyme from cells grown on maltose		
		Substrate					
Added fructose	Product	Sucrose	Methyl α-glucoside	Maltose	Sucrose	Methyl α-glucoside	Maltose
Without	Free glucose	40·0	37·1	0	32·0	3·5	56·4
With	Free glucose	33·2	32·5	0	27·0	3·0	44·8
	Glucosylfructose	5·4	6·2	0	5·7	0·8	8·8

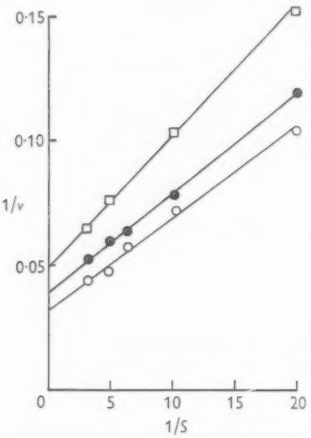


Fig. 1. Inhibition by D-fructose of hydrolysis of sucrose by the yeast α-glucosidase. Reaction mixture contained α-glucosidase extracted from cells grown on methyl α-glucoside and variable concentrations of sucrose and fructose in 0·06 M-sodium phosphate buffer, pH 6·8. After incubation for 30 min. at 37° the amount of liberated glucose was determined iodometrically. *S*, Sucrose concentration (M); *v*, μmoles of glucose/ml. ○, Sucrose alone; ●, with 0·2 M-fructose; □, with 0·4 M-fructose.

Synthesis and isolation of the transglucosylation products

100 ml. of 0·05 M-sodium phosphate buffer, pH 6·6, containing 17·1 g. of sucrose and 18·0 g. of fructose, was incubated for 24 hr. at 30° under toluene with 50 ml. of extract prepared from 10 g. of dry hybrid yeast strain 12836 cells previously grown on methyl α-glucoside. After inactivation by heating for 3 min. in a boiling-water bath, the solution was cooled and brought to pH 4·5 by addition of acetic acid. Invertase concentrate (5 ml.) was added and the solution incubated for 24 hr. at room temperature. This treatment caused total hydrolysis of residual sucrose without the accumulation of any transfructosylation products. To remove free hexose, the solution was treated with 15 g. of freshly packed *Torulopsis glabrata* cells for 10 hr. with shaking at 30°. Cells were removed by centrifuging and the solution was concentrated to a syrup, which on chromatographic examination was found to be free of hexoses and sucrose. The concentrate was loaded on a 25 cm. × 3 cm. Darco G-60–Celite 535 column (Whistler & Durso, 1950) and washed with 4 l. of 1% (v/v) ethanol (fraction I). Ethanol (5%, v/v; 2 l.) yielded fraction II; fraction I was resolved on a 5 cm. × 40 cm. cellulose column (Whatman cellulose powder, standard grade) with 1·5 l. of butanol–ethanol–water (5:1:1, by vol.) as the irrigant. The column contents was extruded and segments at 4–10 cm. and 10–25 cm. from the top surface of the packed column were extracted with hot 20% (v/v) ethanol. The eluents were concentrated and the oligosaccharides were further purified by quantitative band chromatography on sheets of Whatman no. 3 MM filter paper. Yields: 132 mg. of compound *a*; 1370 mg. of *b* (Table 1).

Fraction II contained four oligosaccharides which were separated quantitatively by paper chromatography to yield 49 mg. of *c*, 60 mg. of *d*, 37 mg. of *e* and 12 mg. of *f* (Table 1).

Observation on some properties of the isolated oligosaccharides

Compounds *a*, *b*, *c* and *e* were ketose-containing compounds which on hydrolysis by acid (0·5N-H_2SO_4, 2 hr. at 96°) or by yeast α-glucosidase gave fructose and glucose as sole products of reaction. After reduction of these compounds with sodium borohydride (Abdel-Ahker, Hamilton & Smith, 1951) glucose was found to be the only reducing hexose formed from the reduced compounds on hydrolysis, thus indicating that the fructose moiety is the terminal reducing group in these oligosaccharides. The molar ratio fructose:total hexose was 1:2 for compounds *a*, *b* and *c* and 1:3 for *e*. The molar ratio of reducing aldose:total reducing hexose in the hydrolysates was 1:2 for *a*, *b* and *c* and 2:3 for *e*. Chromatographic examination after partial hydrolysis of *e* (0·1N-H_2SO_4 for 1 hr. at 96°, then neutralization with $BaCO_3$) showed the presence of spots corresponding in mobility to glucose, fructose, isomaltose and isomaltulose.

Compounds *d* and *f* were aldoses which yielded only glucose on hydrolysis by acid or by the α-glucosidase of the haploid yeast.

Values of optical rotation, chromatographic and electrophoretic mobilities of the reference sugars and of the transfer products studied are reported in Table 1. It should be mentioned that the different colour reactions given by the various sugars when tested with spray reagents were highly characteristic. The more outstanding reactions were as follows: a red was afforded with the urea–phosphoric acid reagent by leucrose but by none of the isolated oligosaccharides. With aniline–diphenylamine reagent the compound *a* gave a cherry colour which was given too by 1-β-glucosylfructose but by no other α-glucosylfructoses tested. Maltulose stained deep violet with this reagent whereas isomaltulose yielded an olive–yellow. Among all the tested oligosaccharides compound *a* and 1-β-glucosylfructose alone did not reduce the alkaline aq. triphenyltetrazolium chloride reagent (cf. Feingold *et al.* 1956).

Oxidation of the oligosaccharides with periodate

The method was that of Hough, Woods & Perry (1957). To 6–8 μmoles of sugar dissolved in 3·8 ml. of water, 1 ml. of a fresh solution of 1% (w/v) sodium borohydride was added. After 4 hr. 0·2 ml. of 1·5N-H_2SO_4 was added and 1 ml. samples were taken into 1·4 ml. of water for oxidation at pH 3·6, and a separate series of second 1 ml. samples was taken into 1·4 ml. of 0·25M-sodium phosphate buffer, pH 8·0. A solution (1·6 ml.) containing 15 μmoles of *p*-hydroxybenzaldehyde and 100 μmoles of sodium metaperiodate was added to each tube. After 2 hr. in the dark, the formaldehyde contents of samples were estimated colorimetrically. Reducing sugars were also oxidized by the same procedure but without prior reduction by borohydride. The results showing the amount of formaldehyde formed during oxidation are presented in Table 3.

Phenylosazones of the oligosaccharides

The oligosaccharide [100 mg., except for disaccharides *c* and *d* (20 mg.)], 200 mg. of phenylhydrazine–HCl and 300 mg. of sodium acetate in 2 ml. of water were heated for 45 min. at 96°. The crystals appearing after cooling were washed with cold water and twice recrystallized from methanol–water. Yields were 27–30%. Disaccharide *a* did not yield a phenylosazone under these conditions. For properties of the osazones obtained see Table 1.

Table 3. *Formaldehyde yields afforded by oxidation of oligosaccharides with metaperiodate at different* pH *values with or without prior reduction by borohydride*

Values indicate moles of formaldehyde liberated per mole of carbohydrate. Numbers in parentheses give the expected theoretical values.

Sugar	Without reduction of sugar (oxidation at pH 8·0)	With prior reduction of sugar: Oxidation at pH 3·6	With prior reduction of sugar: Oxidation at pH 8·0
Maltose	2·10 (2)	1·94 (2)	2·85 (3)
Maltulose	—	1·84 (2)	2·86 (3)
Isomaltose	0·00 (0)	0·97 (1)	1·08 (1)
Isomaltulose	0·88 (1)	0·95 (1)	0·92 (1)
Maltotriose	2·70 (3)	1·98 (2)	3·72 (4)
Isomaltotriose	0·00 (0)	0·94 (1)	1·02 (1)
1-β-Glucosylfructose	0·77 (1)	0·95 (1)	0·97 (1)
Transfer products isolated:			
a	0·71	0·91	1·09
b	0·90	1·04	1·00
c	—	1·86	2·68
d	0·00	0·92	1·06
e	—	0·92	0·96

Oxidation of phenylosazones by periodate. The osazone (26mg.) was dissolved in 5 ml. of aq. 66 % (v/v) ethanol and 0·25 ml. of a solution containing 42·8 mg. of sodium metaperiodate was added (one-fifth of these amounts was used for compounds *c* and *d*). The osazones prepared from isomaltose, isomaltulose, melibiose and disaccharides *b* and *d* yielded a precipitate of orange needles of mesoxyaldehyde-phenylosazone which was washed with ethanol and dried; yields were 77–83 %; m.p. 198–200° (Chargaff & Magasanik, 1947; Stodola *et al.* 1956). The osazones prepared from maltose, maltulose, turanose and disaccharide *c* did not yield this product even after incubation with the oxidant for several hours.

DISCUSSION

The observation that the inhibition exerted by fructose on the enzymic hydrolysis of α-glucosides is of a non-competitive type for the α-glucoside substrate (Fig. 1), and the finding that the deficit in the amount of free glucose liberated in the presence of fructose can be accounted for by the appearance of transfer products, lead obviously to the conclusion that the inhibitory effect of fructose on glucose liberation does not involve any decrease in the rate of α-glucoside cleavage but is due to competition of fructose with water for glucosyl units transferred by the enzyme.

It has been shown (Avigad, 1958) that D-glucose and maltose act as competitive inhibitors of the hydrolyses of α-glucosides by the hybrid yeast α-glucosidase and these aldoses are but poor acceptors for the α-glucosyl units transferred by the enzyme. In addition, no exchange reaction could be shown between [^{14}C]glucose and an α-[^{12}C]glucoside serving as substrate, when incubated together with this α-glucosidase. These results, at least in respect of the α-glucosidase studied, do not support the suggestion that fructose inhibits the hydrolysis because it can attach itself to the enzyme in the same manner as does D-glucose or maltose (Gottschalk, 1950).

The hybrid-yeast α-glucosidase used in the present study should be regarded as having variable substrate specificity, the latter being a property subject to alterations which are controlled both by the genotype and the nature of the growth sugar (Avigad, 1958). It was found (Table 2) that fructose was able to serve as an acceptor for the transferred glucosyl group in divers enzyme systems obtained from cells grown on different inducers and possessing a different substrate specificity. This transglucosylation in the different cases, which resulted in the formation of the same products and in the same quantitative relationship to the amount of substrate cleaved, is consistent with the concept that the specificity of transfer to fructose is a constant property of the enzyme and that a single α-glucosidase is present though the substrate specificity can vary.

The hydrolysis of sucrose by the hybrid-yeast α-glucosidase reflects a series of reactions affording a rather complicated picture. The liberated glucose serves as a competitive inhibitor of sucrose hydrolysis whereas it serves only as a poor acceptor for glucosyl units and cannot interchange with the α-glucosyl moiety of sucrose. In addition, the liberated fructose serves as an acceptor for α-glucosyl units removed from the sucrose molecule.

The values of optical rotation, chromatographic and electrophoretic mobility, the characteristics of the phenylosazones formed, the periodate oxidation studies and other analytical data obtained prove unambiguously that the identity of the isolated oligosaccharides is as follows: compound *a*, 1-α-glucosylfructose; *b*, 6-α-glucosylfructose (isomaltulose); *c*, 4-α-glucosylfructose (maltulose); *d*, 6-α-glucosylglucose (isomaltose); *e*, 6^G-α-glucosylisomaltulose (isomaltotriulose); *f*, 6^2-α-glucosylisomaltose (isomaltotriose).

Other transfer products, probably at the level of tri- and tetra-saccharides, were also observed in the chromatographic examination of the reaction-mixture concentrates but they could not be studied owing to their presence in barely detectable amounts.

Isomaltulose was the product isolated in largest quantities. 1-α-Glucosylfructose was obtained in quantities of about one-tenth that of isomaltulose, whereas maltulose was found only in minute amounts. Isomaltotriulose was probably formed by transglucosylation to isomaltulose. Isomaltose and isomaltotriose were probably formed when the glucose liberated served as the initial acceptor. The primary alcoholic groups of D-fructose served preferentially as acceptors in this transfer system; C-6 of fructose exhibited the highest acceptor activity, i.e. the sugar was probably in the furanose form.

Preferential transfer of α-glucosyl units to primary alcoholic groups in aldose acceptors is known to occur in many systems of α-glucosidases from moulds (cf. review by Edelman, 1956) or from *Schizosaccharomyces pombe* (Shibasaki & Aso, 1954). In comparison it should be mentioned that Yasumura (1954) described maltulose and turanose as products of transglucosylation to fructose catalyzed by brewer's-yeast extract acting on sucrose. Weidenhagen & Lorenz (1957) have isolated a bacterium which formed isomaltulose ('palatinose') after contact with sucrose, and Stodola, Sharpe & Koepsell (1954) mention isomaltulose as one of the products formed in small quantities by the action of dextransucrase on

sucrose. It might also prove interesting to study the acceptor specificity of fructose in other known systems of α-glucosidases like that of aphids (which can transfer α-glucosyl to C-3 of fructose in the sucrose molecule with the formation of melezitose; Bacon & Dickinson, 1957) and that of honey α-glucosucrase (White & Maher, 1953).

SUMMARY

1. The inhibitory effect of D-fructose on hydrolysis of α-glucosides by α-glucosidase of a hybrid yeast is non-competitive for the α-glucoside. The fructose competes with water for the α-glucosyl units transferred by the enzyme.

2. Products of transglucosylation to fructose were isolated and characterized. Isomaltulose was the main component found whereas 1-α-glucosylfructose was obtained in smaller amounts. Small quantities of maltulose, isomaltose, isomaltotriose and isomaltotriulose were also isolated. Properties of the hitherto unknown 1-α-glucosylfructose are described.

The author wishes to thank Professor S. Hestrin for helpful advice. This investigation was supported by a research grant (E-1949) from the National Institute of Allergy and Infectious Diseases, of the National Institutes of Health, Public Health Service, Bethesda, Md., U.S.A.

REFERENCES

Abdel-Akher, M., Hamilton, J. K. & Smith, F. (1951). *J. Amer. chem. Soc.* **73**, 4691.

Avigad, G. (1958). *Bull. Res. Coun., Israel*, **7A**, 112.

Bacon, J. S. D. & Dickinson, B. (1957). *Biochem. J.* **66**, 289.

Bates, F. J. (1942). *Polarimetry, Saccharimetry and the Sugars*, Circular 440. Philadelphia: National Bureau of Standards.

Chargaff, E. & Magasanik, B. (1947). *J. Amer. chem. Soc.* **69**, 1459.

Corbett, W. M. & Kenner, J. (1954). *J. chem. Soc.* p. 1789.

Edelman, J. (1956). *Advanc. Enzymol.* **17**, 189.

Feingold, D. S., Avigad, G. & Hestrin, S. (1956). *Biochem. J.* **64**, 351.

Foster, A. B. (1953). *J. chem. Soc.* p. 982.

Gottschalk, A. (1950). In *The Enzymes*, vol. 1, part 1, p. 551. Ed. by Sumner, J. B. & Myrbäck, K. New York: Academic Press Inc.

Herzog, R. O. (1913). In *Die Fermente*, 4th ed., p. 987. Ed. by Oppenheimer, C. Leipzig: Thieme Verlag.

Hestrin, S. & Lindegren, C. C. (1950). *Arch. Biochem.* **29**, 315.

Hestrin, S. & Lindegren, C. C. (1952). *Arch. Biochem. Biophys.* **38**, 317.

Hough, L., Jones, J. K. N. & Richards, E. L. (1953). *J. chem. Soc.* p. 2005.

Hough, L., Jones, J. K. N. & Wadman, W. H. (1950). *J. chem. Soc.* p. 1702.

Hough, L., Woods, B. M. & Perry, M. B. (1957). *Chem. & Ind.* p. 1100.

Isaiev, V. J. (1926). *J. Inst. Brew.* **32**, 552.

Jeanes, A., Wilham, C. A., Jones, R. W., Tsuchiya, H. M. & Rist, C. E. (1953). *J. Amer. chem. Soc.* **75**, 5911.

Macleod, M. & Robison, R. (1929). *Biochem. J.* **23**, 517.

Nelson, N. (1944). *J. biol. Chem.* **153**, 375.

O'Dea, J. F. & Gibbons, R. A. (1953). *Biochem. J.* **55**, 580.

Pacsu, E., Wilson, J. E. & Graf, L. (1939). *J. Amer. chem. Soc.* **61**, 2675.

Pazur, J. H. & Budovich, T. (1956). *J. Amer. chem. Soc.* **78**, 1885.

Peat, S., Roberts, P. J. P. & Whelan, W. J. (1952). *Biochem. J.* **51**, xvii.

Peat, S., Whelan, W. J. & Kroll, G. W. F. (1956). *J. chem. Soc.* p. 53.

Roe, J. H., Epstein, J. H. & Goldstein, N. P. (1949). *J. biol. Chem.* **178**, 839.

Schwimmer, S. & Bevenue, A. (1956). *Science*, **123**, 543.

Shibasaki, K. & Aso, K. (1954). *Tohoku J. agric. Res.* **5**, 131.

Stodola, F. H., Sharpe, E. S. & Koepsell, H. J. (1954). *Abstr. Amer. chem. Soc.* **126**, 5D.

Stodola, F. H., Sharpe, E. S. & Koepsell, H. J. (1956). *J. Amer. chem. Soc.* **78**, 2514.

Thompson, A. & Wolfrom, M. L. (1954). *J. Amer. chem. Soc.* **76**, 5173.

Turvey, J. R. & Whelan, W. J. (1957). *Biochem. J.* **67**, 49.

Wallenfels, K. (1950). *Naturwissenschaften*, **37**, 491.

Weidenhagen, R. & Lorenz, S. (1957). *Z. Zuckerind.* **7**, 533.

Whistler, R. L. & Durso, D. F. (1950). *J. Amer. chem. Soc.* **72**, 677.

White, J. W., Eddy, C. R., Petty, J. & Hoban, N. (1958). *Analyt. Chem.* **30**, 506.

White, J. W. & Maher, J. (1953). *Arch. Biochem. Biophys.* **42**, 360.

Wise, C. S., Dimler, R. J., Davis, H. A. & Rist, C. E. (1955). *Analyt. Chem.* **27**, 33.

Wolfrom, M. L. & Thompson, A. (1957). *J. Amer. chem. Soc.* **79**, 4212.

Yasumura, A. (1954). *J. Jap. biochem. Soc.* **26**, 200.

Yemm, E. W. & Willis, A. J. (1954). *Biochem. J.* **57**, 508.

Further Studies on the Metabolism of the (+)-*anteiso*-Acids, (+)-12-Methyltetradecanoic Acid and (+)-14-Methylhexadecanoic Acid, in the Rat

By T. GERSON, F. B. SHORLAND
Fats Research Laboratory, Department of Scientific and Industrial Research, Wellington, New Zealand
Y. ADAMS AND M. E. BELL
Nutrition Research Department, Medical School, University of Otago, Dunedin, New Zealand

(*Received* 14 *April* 1959)

Recently, Livingston, Bell, Shorland, Gerson & Hansen (1957) found that Wistar rats on a fat-free diet supplemented with 0·1 g. of C_{17} *anteiso*-[(+)-14-methylhexadecanoic] acid/rat/week excreted 10% and stored a similar amount which was partially converted into the C_{15} *anteiso*-acid. The remaining 80% was metabolized to substances other than *anteiso*-acids.

In the present work the object was to study the storage, excretion and interconversion of (+)-*anteiso*-acids given at higher levels than previously. For this purpose the dosage of (+)-14-methylhexadecanoic acid was increased to as much as 3 g./rat/week, and other rats were fed with up to 0·6 g. of (+)-12-methyltetradecanoic acid/rat/week. It was hoped to show in this way the formation of (+)-14-methylhexadecanoic acid from (+)-12-methyltetradecanoic acid. In the course of these investigations the opportunity was taken to ascertain what effect, if any, the feeding of (+)-*anteiso*-acids had on the fatty acid composition of the carcass fat.

EXPERIMENTAL

Male weanling Wistar rats were fed *ad lib.* on a fat-free diet as previously described (Livingston *et al.* 1957). Table 1 shows the supplement given to the six groups of rats used in this work. The animals appeared to find these acids unpalatable, and to ensure ingestion they were given in the form of pellets, further food being withheld until the supplement was completely consumed. Twenty-four hours after the last supplement the rats were killed by decapitation, except those of group 5B, which were kept alive for a further period of 9 days. The whole animals of group 4 (see Table 1) were used for fat extraction, but in the other groups the alimentary tract was discarded, as in the experiments already reported (Livingston *et al.* 1957).

The faeces of groups 1–4 were collected for 48 hr. after each supplement. Those of groups 5A and 5B were collected during the whole supplement period, and with group 5B for a further 9 days until the animals were killed. Faeces were freed from hair and food particles by blowing with a jet of air.

The tissues from each group were combined and minced after death and dropped into approximately 2 vol. of 95% ethanol and boiled for 20 min. The faeces were similarly dehydrated with boiling ethanol, the collections for each group being subsequently combined. The ethanol was distilled off and the tissues and faeces were dried *in vacuo*. The fat was extracted as described by Livingston *et al.* (1957).

Samples of the tissue fats, varying in weight from 30 to 50 g., were saponified, the unsaponifiable matter was extracted and the soaps were converted into fatty acids as described by Hilditch (1956*a*). The acids were crystallized three times from 15 vol. of acetone at −30° to separate them into 'solids' and 'liquids', which were then converted into methyl esters. As previously found (Hansen, Shorland & Cooke, 1954), the (+)-*anteiso*-acids remained in the liquid fraction. The esters were distilled in a fractionating column previously described (column E, Shorland, 1952).

The fatty acid composition of groups 1 and 5A were calculated as described by Hilditch (1956*b*), the necessary allowances for (+)-*anteiso*-acids present being made. The latter, when present in the methyl ester fractions from the 'liquids', were located from their saponification equivalents and quantitatively estimated from their optical rotations (cf. Livingston *et al.* 1957), the values reported by Milburn & Truter (1954) being used for the specific rotations of the pure substances. The (+)-*anteiso*-acids in the faecal lipids

Table 1. *Details of the dietary amounts of* (+)-anteiso-*acids given*

Group no.	No. of rats	Supplement			Duration of supplementary feeding (days)	Age at death (weeks)
		Acid	Wt. (g.)	Milli-equiv./rat		
1	11	—	—	—	—	21
2	5	C_{15}	0·1	0·39 (weekly)	63	21
3	5	C_{15}	0·6	2·34 (weekly)	63	21
4	5	C_{17}	0·6	2·22 (weekly)	49	18
5A	3	C_{17}	0·5	1·85 (daily)	6	20
5B	3	C_{17}	0·5	1·85 (daily)	6	20

Table 2. *Storage, conversion and excretion of* (+)-anteiso-*acids expressed as a molar percentage of the dietary amounts given*

Group* no.	(+)-*anteiso*-Acids given	Total supplement per group (milli-equiv.)	Deposited in carcass (%)	Converted into other *anteiso*-acids (%)	Excreted in faeces (%)	Metabolized to substances other than *anteiso*-acids % (difference)
1	—	Nil	0·0	0·0	0·0	0·0
2	C_{15}	17·55	2·1	0·7	9·5	87·8
3	C_{15}	105·30	2·1	0·4	2·3	95·2
4	C_{17}	77·70	2·1	0·6	2·8	94·5
5A	C_{17}	33·30	7·8	2·7	1·0	88·5
5B†	C_{17}	33·30	9·9	2·2	1·1	86·8

* See Table 1.
† Faeces were collected for 9 days after the last supplement but contained no detectable amounts of (+)-*anteiso*-acids.

Table 3. *Comparison of fatty acid compositions (weight %) of rats fed on* (+)-14-*methylhexadecanoic acid and controls*

	Saturated acids						Unsaturated acids			
Group* no.	C_{14}	C_{15} (+)-*anteiso*	C_{16}	C_{17} (+)-*anteiso*	C_{18}	C_{20}	C_{14}	C_{16}	C_{18}	C_{20-22}
1	2·27	0·00	27·97	0·00	6·93	0·14	0·93 (−2·0, H)†	14·19 (−2·0, H)	45·70 (−2·1, H)	1·87 (−6·0, H)
5A	1·14	0·69	27·58	2·31	2·77	1·31	0·85 (−2·0, H)	15·39 (−2·0, H)	46·68 (−2·1, H)	1·28 (−6·0, H)

* See Table 1. † Mean unsaturation in terms of hydrogen.

were determined after removal of the unsaponifiable matter and bleaching of the recovered acids with animal charcoal. It was assumed that the faecal (+)-*anteiso*-acids were the same as those administered.

RESULTS AND DISCUSSION

In Table 2 are shown the weights of (+)-*anteiso*-acids in the tissues and faeces, from which the extent of storage, conversion into other (+)-*anteiso*-acids and excretion are calculated. The results given show that the C_{15} and C_{17} (+)-*anteiso*-acids are deposited in similar amounts when given at the same level. The amounts of (+)-*anteiso*-acids found were similar in the whole animals of group 4 and in the animals of group 5 whose alimentary tracts had been removed. It would appear therefore that these acids are not stored to any appreciable extent in the alimentary tract. The amounts of C_{15} acid converted into C_{17} acid, and vice versa, are very similar. The behaviour of branched-chain fatty acids therefore does not resemble that of normal fatty acids, in which the lower members (below C_{16}) are less readily deposited than their higher homologues. Thus when decanoic acid is fed to pigs no appreciable amounts are incorporated into the depot fats, whereas dietary lauric acid is deposited to a much smaller extent than myristic acid (Shorland & de la Mare, 1945). It appears that the relative metabolic properties of fatty acids, such as the rate of synthesis and breakdown, determine to a large extent the composition of the depot fat. As storage and interconversion are similar for C_{15} and C_{17} (+)-*anteiso*-acids, it is not surprising that these occur in similar amounts in natural fats, e.g. ewe-milk fat (Gerson, Shorland & Barnicoat, 1958) with 0·41% of C_{15} and 0·45% of C_{17} (+)-*anteiso*-acid and lamb-caul fat (F. B. Shorland, unpublished results), which contains 0·37% of C_{15} and 0·36% of C_{17} (+)-*anteiso*-acid.

Pihl, Bloch & Anker (1950) found that the carcass fatty acids of rats maintained on a fat-free diet had a half-life period of 18 days. As stated above, groups 5A and 5B received identical treatment except that group 5B was maintained for 9 days after the last supplement whereas the rats in group 5A were killed at this stage. A comparison of these groups thus provides a measure of the rate of disappearance of the (+)-*anteiso*-acids. This shows that the rate of turnover of (+)-*anteiso*-acids in rats is much slower than that of the normal fatty acids. In a recent experiment (T. Gerson, J. C. Hawke, F. B. Shorland & W. H. Melhuish, unpublished results) it was found that isotopically-labelled acetate is incorporated into *anteiso*-acids more slowly than into normal acids, thus further demonstrating the slow metabolism of (+)-*anteiso*-acids.

Table 3 shows the fatty acid compositions of groups 1 and 5A and indicates that the feeding of (+)-*anteiso*-acids does not affect appreciably the composition apart from the inclusion of (+)-*anteiso*-acids. However, the reduced amount of stearic acid in group 5A as compared with group 1 may be significant.

SUMMARY

1. Supplements of (+)-12-methyltetradecanoic acid and (+)-14-methylhexadecanoic acid to a fat-free diet did not materially alter the fatty acid composition of the depot fats of Wistar rats.

2. The weights of (+)-12-methyltetradecanoic acid and (+)-14-methylhexadecanoic acid deposited in the carcass under identical conditions of supplementation were proportional to the amounts of supplement given, and the degree of interconversion of these acids was similar, thus affording an explanation for their occurrence in the same proportions in animal fats.

3. The metabolic turnover of (+)-*anteiso*-acids was shown to be considerably slower than that of normal fatty acids.

REFERENCES

Gerson, T., Shorland, F. B. & Barnicoat, C. R. (1958). *Biochem. J.* **68**, 644.

Hansen, R. P., Shorland, F. B. & Cooke, N. J. (1954). *Biochem. J.* **57**, 397.

Hilditch, T. P. (1956*a*). *The Chemical Constitution of Natural Fats*, 3rd rev. ed., p. 572. London: Chapman and Hall Ltd.

Hilditch, T. P. (1956*b*). *The Chemical Constitution of Natural Fats*, 3rd rev. ed., pp. 586–91. London: Chapman and Hall Ltd.

Livingston, M., Bell, M. E., Shorland, F. B., Gerson, T. & Hansen, R. P. (1957). *Biochem. J.* **65**, 438.

Milburn, A. M. & Truter, E. V. (1954). *J. chem. Soc.* p. 3344.

Pihl, A., Bloch, R. & Anker, M. S. (1950). *J. biol. Chem.* **183**, 441.

Shorland, F. B. (1952). *J. appl. Chem.* **67**, 447.

Shorland, F. B. & de la Mare, P. B. D. (1945). *J. agric. Sci.* **35**, 33.

The *N*-Terminal Amino Acid Residues of Gelatin

4. CHAIN WEIGHT AND RIGIDITY RELATIONSHIP IN FRACTIONATED GELATINS*

BY A. COURTS

The British Gelatine and Glue Research Association, 2a Dalmeny Avenue, London, N. 7

(*Received* 9 *June* 1959)

The relations between the macroscopic properties (solution viscosity, gel rigidity, melting point, etc.) of the gelatin–water system and the molecular size, weight, shape and chemical structure of the gelatin molecules remains as the central problem of research in this field.

Pouradier & Venet (1952) showed that an equation of the type $\eta = KM_n^{\alpha}$ exists between the intrinsic viscosity η (or the equivalent limiting viscosity number, LVN) and the number-average molecular weight, M_n, determined osmotically on fractions of gelatin. Whereas K and α were each the same for two alkali-processed gelatins, they were different for an acid-processed gelatin. The evidence for multichain gelatin molecules (Courts & Stainsby, 1958) introduces a further factor into considerations of molecular structure and suggests that the 'molecular-weight' values calculated from assay of N-terminal residues (Courts, 1954*a*, *b*; 1955) should be more correctly termed 'chain weight' (C_n).

Courts & Stainsby (1958) have shown that, in accordance with a multichain molecular model, there is no direct connexion between the following pairs of quantities for fractions of an alkali-processed gelatin: (1) Weight-average molecular weight (M_w) and C_n. Thus C_n for high-grade gelatin fractions has the approximately constant value of 70 000, whereas M_w may vary from 70 000 to 350 000. (2) C_n and LVN. This was demonstrated experimentally but also must arise as an automatic consequence of the relation between M_n and LVN and (1) above, provided that reasonable homogeneity of fractions has been secured.

The picture for acid-processed gelatin fractions is not so readily interpreted. Whereas the value of C_n at 92 000 for the higher fraction is greater than in the corresponding alkali-processed series, M_w at 194 000 is somewhat lower. Since for single chains M_w is greater than C_n unless the fractions are completely homogeneous, branching must be regarded as probable rather than proved. In addition, for the lower fractions of the acid-processed gelatin, M_w is less than C_n, suggesting that some gelatin chains occur with no free α-amino groups. This could arise through blocking with some substance which the alkali process removes. The lack of end-groups in native collagen could be regarded as supporting this view. If a similar proportion of blocked groups occurred in the larger molecules it would explain the figure C_n 92 000

* Part 3: Courts (1955).

compared with 60 000 for alkali-processed gelatin fractions. Alternatively, the blocking could arise as suggested by Landucci, Pouradier & Durante (1958), who find some of the *N*-terminal groups in gelatin to be bound to certain aldehydes. The aldehydes are said to be destroyed in alkali. The absence of a formal relationship between the rigidity of a gelatin gel and LVN was suggested by Stainsby, Saunders & Ward (1954). Detailed study of degraded gelatins by Saunders & Ward (1955, 1958) showed that the low-temperature rigidity for fractions of a single gelatin is approximately constant, if the LVN is in excess of about 40, but for fractions with LVN below this figure the rigidity decreases sharply to zero. They also showed that the value of the constant rigidity obtained above LVN 40 depended on the gelatin used, and could be varied by neutral or alkaline degradation.

In view of the lack of a unique relation between rigidity and LVN (or molecular weight) it was possible that the gel rigidity might be determined by the average chain length (C_n) of component chains of the molecules. This aspect has been studied in the present work.

Methods of gelatin preparation were adopted to give a series of gelatins with marked differences in high-molecular-weight rigidity (i.e. LVN greater than 40). A partial fractionation of the gelatins, by the alcohol coacervation procedure of Stainsby (1954), was carried out to remove the especially high- and the especially low-molecular-weight fractions from a group of gelatin samples prepared from a similarly treated collagen. The rigidity, end-group and LVN values of these were then determined.

In view of the importance often attached to the interaction of mucopolysaccharides and collagen, an examination has been carried out of the mucopolysaccharide content of certain gelatins and gelatin fractions. The results showed that these substances are present as non-active impurities playing no part in the physical properties. This work is reported in the succeeding paper.

MATERIALS AND METHODS

Laboratory-prepared gelatins

The underlying principle of making gelatins with LVN greater than 40 was to subject ossein (the bone collagen used by Courts, 1958, 1960) to mild pretreatments, followed by several extractions of short duration in water at successively higher temperatures (for extraction conditions, see Table 1).

Pretreatment with calcium hydroxide. Ossein (600 g.) was treated with a suspension of $Ca(OH)_2$ (250 g.) in water (4 l.) for 16 days at 20°, was then washed in running water for 8 hr., soaked in 0·01 N-acetic acid overnight and finally washed to pH 7·0 with tap water.

Pretreatment with sodium hydroxide. Sodium hydroxide (0·8 N) may replace the $Ca(OH)_2$ as pretreating agent if a swelling restrainer, e.g. Na_2SO_4, is present (Ames, 1944; A. G. Ward, unpublished work). Soaking for 2 days was used and washing was carried out as described above.

Pretreatment with sulphuric acid. The Deutsche Gelatine Fabriken (1952) process was followed. To 20% Na_2SO_4 solution (3·5 l.) containing conc. H_2SO_4 (35 ml.) ossein (700 g.) was added and allowed to stand overnight. The mixture was then rapidly heated to 80° and maintained at that temperature for 25 min.; the ossein was then immediately strained off, cooled and washed for 1 day.

Fractionation of gelatin

The method was effectively a bulk precipitation of the fractions 2, 3, 4 and 5 of Stainsby (1954). A solution of gelatin (4%) was maintained at 40±0·25° and treated with propan-2-ol–water (90:10, v/v) to the point of slight coacervation. The coacervate (corresponding to fraction 1) was allowed to settle overnight at 40° and the supernatant poured off. The supernatant was treated at 40° with further propan-2-ol–water to precipitate the bulk of the gelatin, which was then allowed to settle at 40° for 6 hr. The supernatant (corresponding to fraction 6) was poured off and the precipitated gelatin remaining was redissolved at 80° in water (about 10 times the weight of gelatin taken). The solution was cooled from 80° to 4°, allowed to set and the gel cut and dried in a stream of cold air. The yields of this bulk fraction were mostly 50–75% of the gelatin taken.

Measurement of gelatin properties

Modulus of rigidity. This was measured by the method of Saunders & Ward (1953). Values for the modulus at 5·65% (w/w) gelatin concentration were taken at 10° and expressed in dynes/cm.². The experimental work was carried out by Mr S. C. Melnick.

Limiting viscosity number (LVN). The viscosity of a 5·65% (w/w) gelatin solution was measured at 40° in a no. 2 Ostwald-type viscometer. This value was related by a standard graph to dilute solution viscosity at 40° (gelatin

Table 1. *Conditions used for the laboratory-prepared gelatins*

Treatment	Extract	Temp.	Time (hr.)	pH
$Ca(OH)_2$	1	55°	1·5	9·8
	2	65	2	10
	3	75	2·5	10
	4	85	2·5	10
	5	95	2·5	10
	6	100	1	10
	7	100	1	11·5
NaOH	1	55	1	7
	2	65	2·5	7
	3	75	2·5	7
	4	85	2	7
	5	95	2	7
	6	100	0·7	7
H_2SO_4	1	55	1·5	3
	2	65	2	3
	3	75	2	3
	4	85	2	3

concentration at 2·5 mg./ml.), and the LVN was calculated from this as in Courts & Stainsby (1958).

Chain weight (C_n). The preparation of dinitrophenyl gelatin, and the isolation and measurement of *N*-terminal residues, was carried out as in Courts (1954*a*). The total (T) of *N*-terminal residues/100 000 g. of gelatin was used to calculate $C_n = 100\,000/T$.

RESULTS

The physical properties of the laboratory-prepared gelatins are given in Table 2. Most of the samples gave an increased modulus of rigidity and LVN after fractionation as a result of the elimination of impurities and degraded gelatin.

Both the alkaline pretreatments used on ossein led to a series of gelatins with a range of LVN values quite suitable for the present study. The acid pretreatment did not meet this requirement and this particular study was not pursued.

The *N*-terminal residue values, before and after fractionation, are given in Table 3 for the gelatins prepared with the sodium hydroxide pretreatment, with C_n calculated from their totals. C_n has been increased by the fractionation in all instances, as would be expected. Corresponding measurements are given in Table 3 for gelatins prepared with the calcium hydroxide pretreatment and subsequently fractionated.

Table 4 shows 13 bulk-fractionated alkali-processed gelatins in declining order of rigidity with the related C_n and LVN values. There is no correlation between any two of these columns.

DISCUSSION

The 'rigidity plateau' of Saunders & Ward (1955) describes, for a series of fractions from a single gelatin, a condition of approximately constant gel rigidity with changing LVN, holding for fractions with LVN greater than 40. It seemed plausible from Table 5 that, since C_n was also approximately constant for the series of fractions giving the 'rigidity plateau', C_n could be directly related to rigidity. The testing of a relationship of this type required the preparation of a series of gelatins embodying widely varying rigidity values, all with LVN greater than 40. A similar preparative history would be an additional requirement for the gelatins. These conditions have been met by giving a short alkaline pretreatment to ossein, followed by a series of extractions in water either at pH 7·0 or a mildly alkaline pH value.

In view of the independence of rigidity and molecular weight for high-viscosity gelatins, it was not necessary to undertake close fractionation. Instead the samples were rendered more homogeneous and free of much of the impurity by removing the top 10–20% and the bottom 20–30%. This technique is successful in removing much of the low-molecular-weight protein, as shown by the general enhancement of C_n, LVN and rigidity-modulus values by the fractionation.

It is evident that the C_n–rigidity relationship, which was suggested by a limited series of fractions of a single gelatin, all at a single value of rigidity, does not hold when tested over a wide range of

Table 2. *Properties of the laboratory-prepared gelatins*

Values based on ash-free gelatin at 15% moisture content.

Treatment	Extract	Gelatin yield (% of ossein taken)	Gelatin fraction obtained (% of gelatin yield)	10^{-3} × Rigidity (dynes/cm.²)		LVN	
				Unfractionated	Fractionated	Unfractionated	Fractionated
$Ca(OH)_2$	1	4·1	37	—	94	48	48
	2	5·4	73	77	89	40	43
	3	9·4	75	70	76	48	51
	4	18·7	56	55	71	53	54
	5	35·6	60	36	51	54	62
	6	5·9	54	33	43	75	81
	7	9·2	49	16	26	43	45
NaOH	1	5·4	60	89	99	48	52
	2	19·4	65	75	95	52	53
	3	16·4	71	74	78	64	76
	4	20·2	44	66	66	75	81
	5	26·7	55	53	65	85	68
	6	6·5	50	33	49	74	82
H_2SO_4	1	23	50	89	104	44	41
	2	20	50	79	95	44	59
	3	22	54	67	86	41	56
	4	12	—	—	—	—	—

Table 3. *N-terminal amino acid residues and C_n of the gelatins*

Values are given as moles/100 000 g. of gelatin.

	$Ca(OH)_2$ process							NaOH process						
	Fractionated gelatins							Fractionated gelatins						Unfractionated gelatins
Liquors ...	1	2	3	4	5	6	7	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
Glycine	0·51	0·57	0·57	0·67	0·70	0·57	0·77	0·76	0·67	0·68	0·74	0·87	0·87	0·88	0·68	0·93	0·77	1·24	1·14						
Serine	0·19	0·14	0·16	0·12	0·12	0·17	0·18	0·12	0·10	0·12	0·16	0·15	0·18	0·12	0·11	0·14	0·10	0·16	0·24						
Threonine	0·17	0·12	0·12	0·15	0·20	0·12	0·17	0·12	0·12	0·10	0·17	0·11	0·14	0·10	0·13	0·16	0·19	0·10	0·15						
Alanine	0·13	0·07	0·08	0·11	0·07	0·08	0·10	0·08	0·06	0·07	0·10	0·09	0·07	0·06	0·08	0·18	0·20	0·09	0·16						
Aspartic acid	0·10	0·12	0·10	0·12	0·12	0·13	0·20	0·11	0·09	0·07	0·09	0·12	0·08	0·10	0·09	0·11	0·15	0·12	0·24						
Glutamic acid	0·14	0·18	0·10	0·09	0·08	0·08	0·20	0·09	0·07	0·17	0·09	0·14	0·12	0·10	0·04	0·16	0·12	0·08	0·21						
Others	0·15	0·09	0·12	0·11	0·08	0·08	0·08	0·07	0·07	0·17	0·10	0·13	0·04	0·19	0·06	0·20	0·25	0·20	0·15						
Total	1·39	1·29	1·25	1·37	1·37	1·23	1·70	1·35	1·18	1·38	1·45	1·61	1·50	1·55	1·19	1·88	1·78	1·99	2·39						
$10^{-3}\,C_n$	72	77	80	73	73	81	59	74	85	73	69	62	66	65	84	53	56	50	44						

Table 4. *Summary of the properties of the laboratory-prepared gelatin bulk fractions*

Pretreatment of ossein	Extract	Rigidity (dynes/cm.²)	C_n	LVN
NaOH	1	99 000	74 000	52
NaOH	2	95 000	85 000	53
$Ca(OH)_2$	1	94 000	72 000	48
$Ca(OH)_2$	2	89 000	77 000	43
NaOH	3	78 000	73 000	76
$Ca(OH)_2$	3	76 000	80 000	51
$Ca(OH)_2$	4	71 000	73 000	54
NaOH	4	66 000	69 000	81
NaOH	5	65 000	62 000	68
$Ca(OH)_2$	5	51 000	73 000	62
NaOH	6	49 000	66 000	82
$Ca(OH)_2$	6	43 000	81 000	80
$Ca(OH)_2$	7	26 000	59 000	45

Table 5. *Properties of gelatin X fractions**

Fraction	Fraction yield (%)	LVN	Rigidity (dynes/cm.²)†	C_n
2	31·2	88	63 000	55 000
3	25·3	65·5	68 000	70 000
4	21·0	51	71 000	70 000
5	18·0	40	75 000	63 000
2/2‡	8·5	92	64 000	60 000

* From Courts & Stainsby (1958).
† Calculated from Saunders & Ward (1958).
‡ Second fraction of fraction 2 refractionated.

rigidity moduli corresponding to the different 'rigidity plateaus' of a number of gelatins.

It is also clear that the absence of relationship between C_n and LVN, as shown by Courts & Stainsby (1958), is confirmed over the wide range of LVN values between 43 and 82. The C_n measurements showed only a narrow range of values, between 59 000 and 85 000, with half the samples giving C_n 71 000 ± 4 000, i.e. within the experimental error of the mean value for the series of gelatins. This type of constancy for C_n, which is similar to the value reported for a series of gelatin fractions (Courts & Stainsby, 1958), is probably accounted for by the concept of branched-chain molecules discussed in that paper. The mean value is also consistent with the 'parent gelatin' subunit of molecular weight 70 000, proposed by Gallop (1955).

No obvious relationship occurs between the rigidity modulus and LVN for the set of gelatins, as would be expected from previous work (Saunders & Ward, 1955); in fact, two gelatins with similar LVN values (43 and 45) were shown to have rigidity moduli as divergent as 89 000 and 26 000 dynes/cm.². The modulus of rigidity of a gelatin gel, with the conditions of measurement used in this paper, is therefore independent of both the molecular weight and the individual gelatin-chain dimensions.

SUMMARY

1. The preparation of a series of gelatins with high molecular weight is described. These were purified by partial fractionation.

2. The modulus of rigidity of the gelatin gels prepared in this way is independent of the molecular weight, as indicated by the limiting viscosity number, and of the gelatin-chain dimensions, as indicated by the *N*-terminal amino acid residues.

I am most grateful to Dr G. Stainsby for advice on the alcohol fractionation of gelatin and the interpretation of the viscosity results. This paper is published by permission of the Director and Council of The British Gelatine and Glue Research Association.

REFERENCES

Ames, W. M. (1944). *J. Soc. chem. Ind., Lond.*, **63**, 5.
Courts, A. (1954*a*). *Biochem. J.* **58**, 70.
Courts, A. (1954*b*). *Biochem. J.* **58**, 74.
Courts, A. (1955). *Biochem. J.* **59**, 382.
Courts, A. (1958). *Recent Advances in Gelatin and Glue Research*, p. 145. Ed. by Stainsby, G. London: Pergamon Press Ltd.
Courts, A. (1960). *Biochem. J.* (in the Press).
Courts, A. & Stainsby, G. (1958). *Recent Advances in Gelatin and Glue Research*, p. 100. Ed. by Stainsby, G. London: Pergamon Press Ltd.
Deutsche Gelatine Fabriken (1952). Brit. Patent 682,829.
Gallop, P. (1955). *Arch. Biochem. Biophys.* **54**, 501.
Landucci, J. M., Pouradier, J. & Durante, M. (1958). *Recent Advances in Gelatin and Glue Research*, p. 62. Ed. by Stainsby, G. London: Pergamon Press Ltd.
Pouradier, J. & Venet, M. (1952). *J. Chim. phys.* **49**, 85.
Saunders, P. R. & Ward, A. G. (1953). *Proc. 2nd int. Congr. Rheology*, p. 284.
Saunders, P. R. & Ward, A. G. (1955). *Nature, Lond.*, **176**, 26.
Saunders, P. R. & Ward, A. G. (1958). *Recent Advances in Gelatin and Glue Research*, p. 197. Ed. by Stainsby, G. London: Pergamon Press Ltd.
Stainsby, G. (1954). *Disc. Faraday Soc.* **18**, 288.
Stainsby, G., Saunders, P. R. & Ward, A. G. (1954). *J. Polym. Sci.* **12**, 325.

The Distribution of Mucoprotein in Gelatins and Fractionated Gelatins

By A. COURTS
The British Gelatine and Glue Research Association, 2a Dalmeny Avenue, London, N. 7

(*Received 9 June* 1959)

A method has been developed for showing the redistribution of mucoprotein brought about by fractionating gelatin, and for demonstrating the differences in mucoprotein content of gelatins inherent in the different connective tissues, species and processes used in their preparation.

The estimation of mucoprotein in gelatin was by a relatively rapid method which does not require extensive acid hydrolysis. The isolation procedure was based on Westphal (1952), in which mucopolysaccharides are separated from proteins by partition in the phenol–water system. With certain gelatins the present author found that the aqueous phase retained material giving a violet with acid diphenylamine (Dische, 1929), which shows identity from absorption curves with colours developed by serum mucoproteins and sialic acid (Werner & Odin, 1952) and urinary mucoproteins (Anderson & Maclagen, 1955).

MATERIALS AND METHODS

Monosaccharides

Commercially available products were used: D-glyceraldehyde, D-erythrose, D-galacturonic acid (L. Light and Co. Ltd.); D-glucose, D-mannose (British Drug Houses Ltd.); L-arabinose, L-xylose, salicin (T. Kerfoot and Co.); D-glucurono-lactone (Corn Products Refining Co., New York); sedoheptulose anhydride (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.).

Collagens

Ox-bone collagen (ossein): the ossein was of the same batch as described in Courts (1958, 1959*a*). Ox-tendon collagen: tendon Achilles of Argentinian cattle was used. This is a commercially available material which has been sun-dried to about 20% moisture content. Ox-skin collagen: the skin, freshly flayed, had the hair removed by shaving but was otherwise untreated.

Gelatins

Intact gelatins: commercial gelatins were used, except where indicated; for the type of gelatin, see Table 1. Gelatins subjected to fractionation: gelatin X: this was the alkali-processed gelatin (Laboratory sample no. 127) used by Saunders & Ward (1958) and Courts & Stainsby (1958). Gelatin D: this was the acid-processed gelatin (Laboratory sample no. 111) used by Courts & Stainsby (1958).

Fractionation of gelatin

Procedure 1: this was the method of Stainsby (1954) in which a series of six fractions was prepared with ethanol as the coacervant.

Procedure 2: the method was effectively a bulk precipitation of the fractions 2–5 of procedure 1. Details are given in the preceding paper (Courts, 1959).

Estimation of mucoprotein

Partition of carbohydrate and protein. Westphal (1952) suggested that chondroitin sulphate may be isolated from cartilage by heating in a phenol–water system whereby, on cooling, the chondroitin sulphate appears in the aqueous phase and the protein in the phenolic layer. Adapting this method to gelatin, the present author found that cold aq. 50% (w/w) phenol was a satisfactory reagent for isolating the carbohydrate moiety from a gelatin sample. The strong hydrogen-bond-breaking activity of phenol solutions liquefies and precipitates gelatin in the cold (Courts, 1958), thus obviating any heating stage and possible thermal degradation of the polysaccharides. The separation of a phenol–water–gelatin system into two phases was improved by a prior digestion of the gelatin with trypsin. The partition was carried out within the range pH 4·0–9·0.

Colorimetric estimation. The aqueous phase was examined with the acid diphenylamine reaction of Dische (1929), as used by Anderson & Maclagen (1955) for urinary mucoproteins. The colour densities were related to a mucoprotein standard, which was a preparation extracted from ox tendon by 5% sodium dodecyl sulphate and purified by dialysis.

Reagents. (i) Gelatin: a solution of gelatin (1 g.) in water (20 ml.) was treated with 0·4% crystalline trypsin (Armour Laboratories) (0·2 ml.) at 37° for about 2 hr. (ii) Phenol: phenol (May and Baker Ltd.) (100 g.) was dissolved in water (100 ml.) by warming and the mixture allowed to stand overnight at 20°. The upper layer was discarded. (iii) Diphenylamine: the reagent used was a solution of diphenylamine (1 g.) in acetic acid (90 ml.) and conc. H_2SO_4 (10 ml.), all of A.R. standard.

Procedure. Gelatin (20 ml.) and 20 ml. of phenol were shaken together for 10 sec. and then allowed to stand until two layers separated (1–5 hr., depending upon the gelatin). A portion (5 ml.) of the aqueous phase and 5 ml. of diphenylamine solution were mixed in a stoppered test tube and heated at 100° for 30 min.; the mixture was then allowed to cool slowly. Occasionally a white solid precipitated, which was filtrable after standing for 2–3 hr. The extinction of the violet was measured by the Spekker spectrophotometer (Hilger and Watts Ltd.) with the 604 filter (Ilford Ltd.) at 530 mμ. The colour was not affected by dissolved phenol, hexosamines, glycine, tryptophan, tyrosine, histidine, urea or thiolacetic acid.

Reproducibility is better than 5%. The lower limit of sensitivity is about 1 part in 5000.

Absorption spectra. Extinction measurements on the diphenylamine colour complexes were made within the range 400–900 mμ in a Uvispek spectrophotometer (Hilger and Watts Ltd.). A 2 cm. cell was used.

RESULTS

The diphenylamine reagent appeared to give colour-forming reactions with two distinct classes of compounds. First, nitrates and nitrites gave a green, the absorption spectrum of which showed peaks at 405 and 800 mμ (Fig. 1). The second group, comprising certain carbohydrates and related compounds, yielded colours for simple sugars in the range violet–blue to green, the colour depending upon the structure (see Table 3). The violet given by the reagent and showing an absorption maximum at 530 mμ appeared to be specific for mucoproteins and for sialic acid.

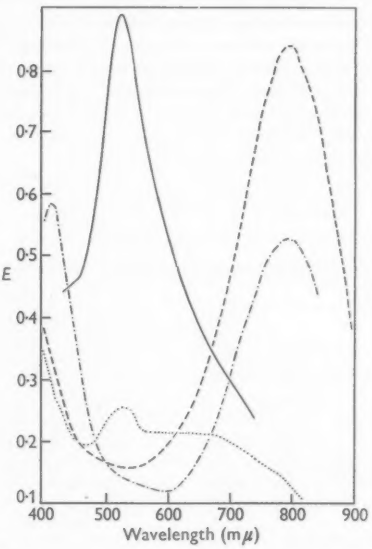


Fig. 1. Absorption curves of the colour complexes of diphenylamine with: ——, *N*-acetylneuraminic acid; ····, acid-processed gelatin; - - - -, alkali-processed gelatin; –·–·–, nitric acid.

Table 1. *Mucoprotein content of different commercial gelatins and some collagens*

Source of gelatin	Pretreatment	Mucoprotein (mg./g. of gelatin)
Ox bone	Acid	15
Ox bone	Acid	19
Rabbit skin	Acid	17
Rabbit skin	Acid	17
Pig skin	Acid	13
Pig skin	Acid	9
Ox skin	Alkali	Trace
Ox skin	Alkali	Trace
Ox skin	Alkali	Trace
Ox bone	Alkali	Trace
Whale skin	Acid	1
Isinglass	Acid	1
Ox bone	Autoclaved	53
Source of collagens		
Ox bone	Demineralized	22
Ox tendon	None	5
Ox skin	None	5

A number of gelatins have been examined with the diphenylamine reagent and the mucoprotein contents are given in Table 1. Those gelatins prepared by an acid process, or by autoclaving ox bone, gave a violet. Gelatins prepared by an alkali process gave greens, generally faint in comparison with the intensities of violet.

The violet complex showed a maximum at 530 mμ on the absorption curve, resembling that developed by the serum mucoproteins (Werner & Odin, 1952) and the urinary mucoproteins (Anderson & Maclagen, 1955). Gelatin D, fractionated by both the procedures 1 and 2, showed the distribution of mucoprotein given in Table 2. It was apparent that the bulk (75–80%) of the mucoprotein was concentrated into the residual fraction.

Table 2. *Distribution of mucoprotein in gelatin D fractions*

For fractionation procedures, see Courts (1959).

		Wt. of gelatin (g.)	Wt. of muco-protein (mg.)	Concn. of mucoprotein (mg./g. of gelatin)
Unfractionated		100	1300	13
	Fraction			
Procedure 1	1	3·8	49	13
	2	32·2	155	5
	3	23·3	42	2
	4	19·1	25	1
	5	15·5	25	2
	6	4·4	Not examined	
Recovery (%) ...		98·3	25	
Procedure 2	First	6·3	82	13
	Bulk	87·5	175	2
	Last	6·2	938	160
Recovery (%) ...		100	94	

The green complex developed by alkali-processed gelatins with diphenylamine gave an absorption maximum at 800 mμ. De-ionization of the gelatin, by the mixed-bed-resin procedure of Janus, Kenchington & Ward (1951), removed the factor able to form the green complex.

Fractionation of gelatin X (see the preceding paper, Courts, 1959) concentrated the factor into

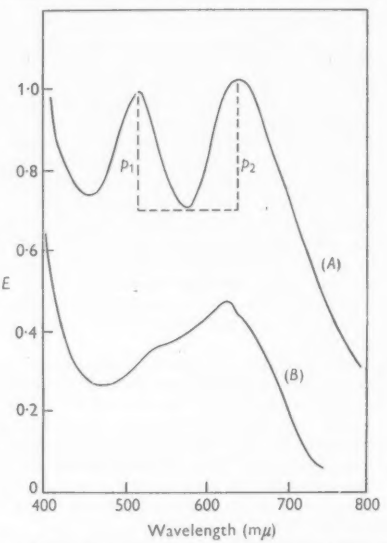


Fig. 2. Absorption curves of the colour complexes of diphenylamine with monosaccharides. (*A*) General curve for C_3–C_6 sugars; the positions and relative heights of p_1 and p_2 are given in Table 3. (*B*) Curve for sedoheptulose.

Table 3. *Colours and absorption maxima of the diphenylamine complex with certain compounds*

Compound	Colour of complex with diphenylamine	First peak [p_1 (mμ)]	Second peak [p_2 (mμ)]	Height (p_1:p_2)
Glyceraldehyde	Green	400	610	1:2
D-Erythrose	Green	430	620	7:1
L-Arabinose	Blue–green	490	650	1:2·4
L-Xylose	Blue–green	490	650	1:2
D-Mannose	Blue	520	650	1:1·1
D-Glucose	Blue	520	650	1:2
Salicin	Blue	520	650	
D-Glucuronic acid	Blue	500	640	1:2·4
D-Galacturonic acid	Blue	500	640	1:1·5
Glucosamine	None	—	—	
Galactosamine	None	—	—	
Sedoheptulose	Blue	—	625	
Folid acid	Green	<400	660	
Pyruvic acid	Green	<400	620	
Nitric acid	Green	405	800	
Sialic acid*	Violet	530	—	
Osseo-mucoprotein	Violet	530	—	

* The compound used was *N*-acetylneuraminic acid.

fraction 6 with the last 3 % of the gelatin. Fig. 2(*A*) represents a general absorption curve given by carbohydrates up to C_6. As will be seen from both Fig. 1 and Table 3, the absorption curve for the green complex showed no resemblance to a triose, tetrose, pentose, hexose or to hexuronic acid; nor did it resemble the curves produced by two other green chromophores, folic acid and pyruvic acid. The curve did, however, correspond with that given by nitric acid.

DISCUSSION

It is probable (Anderson & Maclagen, 1955; Dische, 1958) that the diphenylamine reaction for estimating mucoproteins is, in fact, dependent upon the sialic acid in association with them. The presence of sialic acid in connective tissue has not previously been reported. Other compounds are present in connective tissue, however, which could contribute to the colour density measured at 530 mμ, even though they do not give rise to the violet specific for sialic acid. Thus, Eastoe & Eastoe (1954) did not find free pentoses or hexoses associated with bone tissue, but consider osseo-mucoprotein to contain galactose, a small quantity of mannose, hexuronic acid, galactosamine and glucosamine. Although the hexosamines will not contribute to the colour, the hexoses and hexuronic acid will. It is difficult to obtain an assessment of the last-named compounds, and their contribution to the colour yield may be quite small. It is therefore still preferable to employ mucoprotein as the standard in the diphenylamine reaction rather than sialic acid.

Gelatins prepared by an acid process or by autoclaving retain much of the mucoprotein present in the original collagen. Alkali-processed gelatins, on the other hand, contain relatively little mucoprotein. It is likely (Courts, 1960) that the mucoprotein is almost entirely dissolved away into the alkaline-pretreatment reagents. The diphenylamine reagent therefore provides a rapid method for identifying the two types of gelatin. The chromophoric factor in the alkali-processed gelatins appears to be ionically bound nitrate (or nitrite). It is possible that the large number of free basic groups along the gelatin chains have formed ionic links with those anions made available through the tap water used in the processing. The nitrate–nitrite level in London tap water is of the order of 5 p.p.m. (Metropolitan Water Board, personal communication).

The alcohol-fractionation technique (Stainsby, 1954) has proved useful not only for fractionating gelatin by M_w, but also for removal from the bulk of the gelatin of most of the mucoprotein, inorganic nitrates, ferric salts (A. Courts, unpublished work) and probably other extraneous materials which have some solubility in the alcohol.

Ox bone is a much richer source of mucoprotein than ox tendon or ox skin, and this is most noticeable where the whole bone has been autoclaved in water. Rabbit skin appears to have a higher content of mucoprotein than ox skin.

The colours developed with diphenylamine by monosaccharides from C_3 to C_6 form an interesting family of absorption spectra in the range 400–800 mμ. They each show a twin-peaked curve in which position of the first peak is especially dependent on the number of carbon atoms. The absorption spectrum of a C_7 monosaccharide indicates a merger of the two peaks in a single broad peak with a shoulder.

SUMMARY

1. The carbohydrate moiety of gelatin may be separated for subsequent estimation by partition in water–phenol.

2. A fairly rapid method, this separation being used together with Dische's acid diphenylamine reagent, is described which shows marked differences between acid- and alkali-processed gelatins in their mucoprotein content.

3. The alcohol fractionation of gelatin concentrates the bulk of the non-gelatin impurities into the residual fraction.

4. The colour complexes formed by diphenylamine with monosaccharides from C_3 to C_7 show a family of absorption spectra with two peaks in the range 400–800 mμ. The position of the peaks is closely related to the number of carbon atoms.

I am indebted to Miss Patricia Carroll, Medical Research Council, Mill Hill, for her kind gift of *N*-acetylneuraminic acid.

REFERENCES

Anderson, A. J. & Maclagen, N. F. (1955). *Biochem. J.* **59**, 638.

Courts, A. (1958). *Recent Advances in Gelatin and Glue Research*, p. 145. Ed. by Stainsby, G. London: Pergamon Press Ltd.

Courts, A. (1959). *Biochem. J.* **73**, 596.

Courts, A. (1960). *Biochem. J.* (in the Press).

Courts, A. & Stainsby, G. (1958). *Recent Advances in Gelatin and Glue Research*, p. 100. Ed. by Stainsby, G. London: Pergamon Press Ltd.

Dische, Z. (1929). *Mikrochemie*, **7**, 33.

Dische, Z. (1958). *Chemistry and Biology of the Mucopolysaccharides*, p. 284. Ed. by Wolstenholme, G. E. W. & O'Connor, C. M. London: J. and A. Churchill Ltd.

Eastoe, J. E. & Eastoe, B. (1954). *Biochem. J.* **57**, 453.

Janus, J. W., Kenchington, A. W. & Ward, A. G. (1951). *Research, Lond.*, **4**, 247.

Saunders, P. R. & Ward, A. G. (1958). *Recent Advances in Gelatin and Glue Research*, p. 197. Ed. by Stainsby, G. London: Pergamon Press Ltd.

Stainsby, G. (1954). *Disc. Faraday Soc.* **18**, 288.

Werner, I. & Odin, L. (1952). *Acta. Soc. Med. Upsalien*, **57**, 230.

Westphal, O. (1952). *Z. Naturf.* **73**, 148.

Tissue Fractionation Studies

9. ENZYMIC RELEASE OF BOUND HYDROLASES*

By H. BEAUFAY† and C. de DUVE
Department of Physiological Chemistry, University of Louvain, Belgium

(*Received* 24 *March* 1959)

Numerous earlier publications from this Laboratory (for reviews, see de Duve, 1958, 1959) have been devoted to the characterization of a new group of cytoplasmic particles called lysosomes. In rat liver, lysosomes can be distinguished from mitochondria, though not completely separated from them, thanks to a somewhat slower rate of sedimentation in 0·25 M-sucrose. They behave in freshly isolated preparations as essentially inert little bags which, upon injury by any of a variety of means, release into the medium a collection of soluble hydrolytic enzymes with an acid pH optimum. Amongst these are the five hydrolases studied in this laboratory, acid phosphatase, β-glucuronidase, 'cathepsin' [probably a mixture of cathepsins A, B (Rademaker & Soons, 1957) and C (Finkenstaedt, 1957)], acid ribonuclease (ribonuclease II) and acid deoxyribonuclease (deoxyribonuclease II), to which may be added the arylsulphatases A and B (Viala & Gianetto, 1955; Roy, 1958), phosphoprotein phosphatase (Paigen & Griffiths, 1959) and *N*-acetyl-β-glucosaminidase (Sellinger & Doyen, 1959).

The means so far known to effect the release of lysosomal enzymes depend on mechanical breakage (in a Waring Blendor), osmotic rupture (in media of low tonicity or, more slowly, in solutions of substances such as sodium chloride, potassium chloride or glycerol, which afford only transient osmotic protection), physicochemical disruption (by repeated freezing and thawing or by exposure to surface-active agents such as saponin, deoxycholate or Triton X-100) or some kind of autolytic breakdown (thermal activation) which occurs when the particles are incubated at 37° and which is favoured by a lowering of pH. Sonic vibrations have also proved effective in some preliminary experiments. It is probable that all of these treatments act by causing a primary injury to the particle membrane, which then allows the external substrates to penetrate within the particles as well as the internal enzymes to leak out of them. This accounts for the simultaneous activation and solubilization of the bound hydrolases.

In the present studies, the effects on the state of lysosomal hydrolases of pretreating the particles with various enzyme preparations have been investigated. The mechanism of thermal activation has also been studied. The results, some of which have been reported in a preliminary communication (Beaufay, 1957), are in essential agreement with the membrane hypothesis and demonstrate further that the lysosomal membrane is of lipoprotein nature.

METHODS

All experiments were performed on mitochondrial fractions isolated from rat liver as described by Appelmans & de Duve (1955). Adequate amounts of the mitochondrial preparations, corresponding in most cases to 0·2 g. of fresh liver, were pre-incubated for 10 min. or more at 25° in a total volume of 1 ml. containing 0·25 M-sucrose, 15 mM-glyoxaline hydrochloride buffer, pH 7·4, and the enzymic agent under study together with its activator or inhibitor as required.

The enzymes applied during pre-incubation included: a purified sample of lecithinase (a gift from Dr B. Cinader) isolated from *Clostridium welchii* cultures by fractionation with $(NH_4)_2SO_4$ and dialysis, and furnished as a concentrated solution in aq. 50% (v/v) glycerol; crystalline trypsin, donated by the Novo Laboratories, Copenhagen; crystalline chymotrypsin, prepared from five-times recrystallized chymotrypsinogen according to Kunitz & Northrop (1935) and recrystallized three times; a commercial concentrate of pancreatic enzymes (Absolute Pancreatin powder, Merck); crystalline ribonuclease, purified according to the method of Kunitz (1940) as modified by McDonald (1949) and recrystallized three times; crystalline lysozyme, purchased from the Armour Co. Eastbourne, Sussex. When lecithinase was studied, the medium was supplemented with 4 mM-$CaCl_2$ to activate the enzyme, and special controls were set up, containing either 4 mM-$CaCl_2$ and as much glycerol as was added with the enzyme or the complete active mixture together with 8 mM-ethylenediaminetetra-acetic acid (EDTA), which inhibits the phospholipase completely. When not present in the pre-incubation medium, this inhibitor was added together with the substrate mixtures to prevent the lecithinase from acting during the subsequent enzymic assays. In some of the experiments with trypsin, crystalline soyabean inhibitor (Sigma Chemical Co., St Louis, Mo., U.S.A.) was used in analogous controls.

After pre-incubation, the free and total enzymic activities of the preparations were determined by adding 1 ml. of substrate mixture and incubating further for 10 min. at

* Part 8: Wattiaux, Baudhuin, Berleur & de Duve (1956).

† Chargé de recherches du FNRS.

37°. For the measurement of free activities, the substrate mixtures were made up so as to realize the assay conditions described by Gianetto & de Duve (1955) and by de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955); they contained in addition all the components of low molecular weight present in the pre-incubation medium. As observed before (Berthet, Berthet, Appelmans & de Duve, 1951) and again here in some preliminary experiments, lack of the last-named precaution may be responsible for the osmotic rupture of the particles, if they are permeable to one or more of the omitted components. The methods used for stopping the reactions and performing the subsequent analyses were those described previously (de Duve *et al.* 1955). The total activities were measured as the free activities, but in the presence of 0·1% of Triton X-100 (Rohm and Haas Co., Philadelphia, Pa., U.S.A.), according to the techniques of Wattiaux & de Duve (1956).

The chemical changes suffered during pre-incubation by the particles themselves were followed on separate samples with the aid of the methods described by Beaufay & de Duve (1954).

RESULTS

Enzymic activation

In Table 1 is given a summary of the main results obtained on mitochondrial fractions pretreated with various enzyme preparations. The experiments were generally run in parallel on acid phosphatase and on one other lysosomal hydrolase, with the result that more data are available for the former enzyme than for the others. For obvious reasons, cathepsin could not be measured in mixtures containing large amounts of proteolytic enzymes; nor could the endogenous acid ribonuclease be assayed after treatment with the homologous crystalline enzyme.

As a rule, the total activities of lysosomal hydrolases suffered only minor changes under the influence of the various treatments applied, with the exception of acid deoxyribonuclease, which exhibited considerable variations. The great sensitivity of this enzyme to its ionic environment (Rotherham, Schottelius, Irvin & Irvin, 1956; Shack, 1957) may explain the moderate activation produced by the lecithinase medium, whereas the inhibitions observed in the presence of ribonuclease and lysozyme are probably attributable to the binding of substrate by these basic proteins. We are unable to account for the striking activation of the deoxyribonuclease by trypsin. This phenomenon was found to depend both on the concentration of trypsin and on the duration of pre-incubation and must therefore involve the actual digestion of some material. Whether the latter process modifies the enzyme molecule, destroys an inhibitor or causes an activator to appear has not been ascertained.

Of more immediate interest to the object of the present study are the increases in free activity observed under the influence of several of the enzyme preparations applied. Further data concerning these effects are given in Table 2 and in Figs. 1 and 2.

As already shown by MacFarlane & Datta (1954), treatment of the mitochondrial fractions with *C. welchii* phospholipase in the presence of Ca^{2+} ions caused practically complete activation of the masked acid phosphatase. This effect, which was accompanied by a considerable solubilization of the enzyme (Table 2), was reproduced to only a very small extent by Ca^{2+} ions alone and was completely inhibited by EDTA (Table 1); it could be achieved with much smaller amounts of lecithinase than were used in the experiments of Table 1, and, when rendered partial by the use of submaximum concentrations of phospholipase, was found to parallel the amount of acid-soluble organic phosphate formed (Fig. 1). It may therefore be safely attributed to the hydrolysis of choline-containing phospholipids present in the preparation. Essentially similar findings were made on β-glucuronidase and on the two nucleases, with the exception that the last-named enzymes, though completely activated, were solubilized to a smaller extent. Cathepsin was activated in the same specific manner by phospholipase, but this activation did not exceed a maximum of approximately 40% and was not accompanied by any appreciable solubilization of the enzyme.

Trypsin also caused a specific and important release of the four acid hydrolases which could be studied. This effect was completely suppressed by a slight excess of soya-bean inhibitor (Table 1) and accompanied the initial rapid phase of proteolytic attack (Fig. 2). Chymotrypsin, though similarly able to release the acid hydrolases, was much less effective in this respect than trypsin. Pancreatin powder showed a strong releasing activity, attributable for its larger part to EDTA-sensitive enzymes, presumably lipases or phospholipases or both, partly also to an EDTA-insensitive component, possibly trypsin. Finally, no significant effects were obtained with ribonuclease or lysozyme. The former result contradicts a report by Allard, de Lamirande & Cantero (1955), but these authors did not control the pH of their pre-incubation medium and used a commercial preparation of ribonuclease, which may have been contaminated by proteolytic enzymes.

Thermal activation

The observation that proteolytic enzymes can cause the release of lysosomal enzymes prompted a re-investigation of the possible role played by the particles' own cathepsin in this process as it occurs spontaneously. Such a role has been suspected before on the basis of temperature and pH effects (Berthet *et al.* 1951; Appelmans & de Duve, 1955).

Table 1. *Influence of various enzymic treatments on activity of lysosomal hydrolases*

When more than one experiment has been performed, means are given ± S.E.M.; figures in parentheses: numbers of experiments. APase, acid phosphatase; β-Gase, β-glucuronidase; RNase, acid ribonuclease; DNase, acid deoxyribonuclease.

Pre-incubation at 25° in 0·25 M-sucrose + 15 mM-glyoxaline HCl buffer, pH 7·4						
Addition to pre-incubation medium	Time (min.)	APase	β-Gase	RNase	DNase	Cathepsin
		Total enzymic activities (% of control)				
None	10	100	100	100	100	100
Glycerol (5%) + 4 mM-$CaCl_2$	10	91 ± 3·9 (5)	108	90	128	90
Glycerol (5%) + 4 mM-$CaCl_2$ + lecithinase (1:10)	10	97 ± 4·3 (5)	105	89	125	97
Glycerol (5%) + 4 mM-$CaCl_2$ + lecithinase (1:10) + 8 mM-EDTA	10	104 ± 4·5 (5)	110	87	128	—
Chymotrypsin (10 mg./ml.)	10	93 ± 2·0 (5)*	96	79	105	—
Pancreatin (10 mg./ml.)	10	100 ± 3·1 (2)	—	100	97	—
Pancreatin (10 mg./ml. + 8 mM-EDTA)	10	97	—	—	96	—
Ribonuclease (1 mg./ml.)	10	91 ± 3·5 (6)*	103	—	74	100
Lysozyme (10 mg./ml.)	10	103 ± 4·3 (6)	94	97	47	96
None	30	100	100	100	100	—
Trypsin (1·0 mg./ml.)	30	93 ± 1·5 (5)†	94 ± 2·3 (3)	115 ± 1·7 (2)	229	—
Trypsin (1·0 mg./ml.) + soya-bean inhibitor (1·25 mg./ml.)	30	92	98	—	—	—
Chymotrypsin (10 mg./ml.)	30	87	—	80	—	—
None	60	100	—	100	—	—
Chymotrypsin (10 mg./ml.)	60	88	—	77	—	—
		Free enzymic activities (% of total)				
None	10	11 ± 0·4 (6)	13	15	13	0
Glycerol (5%) + 4 mM-$CaCl_2$	10	14 ± 0·9 (5)*	12	20	8	10
Glycerol (5%) + 4 mM-$CaCl_2$ + lecithinase (1:10)	10	91 ± 3·9 (5)‡	107	90	108	44
Glycerol (5%) + 4 mM-$CaCl_2$ + lecithinase (1:10) + 8 mM-EDTA	10	12 ± 0·8 (5)	10	18	10	6
Trypsin (0·2 mg./ml.)	10	34§	—	—	—	—
Trypsin (1·0 mg./ml.)	10	51§	—	—	—	—
Chymotrypsin (10 mg./ml.)	10	18 ± 3·3 (5)	23	30	27	—
Pancreatin (10 mg./ml.)	10	64 ± 6·0 (2)‡	—	69	61	—
Pancreatin (10 mg./ml. + 8 mM-EDTA)	10	31	—	—	19	—
Ribonuclease (1 mg./ml.)	10	14 ± 1·7 (6)	11	—	6	0
Lysozyme (10 mg./ml.)	10	10 ± 0·6 (6)	14	28	10	2
None	30	12 ± 2·2 (5)	17 ± 8·5 (2)	11	—	—
Trypsin (0·2 mg./ml.)	30	61§	—	—	—	—
Trypsin (1·0 mg./ml.)	30	76 ± 5·7 (5)‡	62 ± 9·0 (3)*	95 ± 8·8 (2)	101	—
Trypsin (1·0 mg./ml.) + soya-bean inhibitor (1·25 mg./ml.)	30	12	19	—	—	—
Chymotrypsin (10 mg./ml.)	30	33	—	53	—	—
None	60	24	—	42	—	—
Chymotrypsin (10 mg./ml.)	60	60	—	74	—	—

* Difference from controls significant at $P < 0·05$. † Difference from controls significant at $P < 0·01$. ‡ Difference from controls significant at $P < 0·001$.
§ Values expressed as percentages of total activity before pre-incubation with trypsin.

Table 2. *Influence of lecithinase on solubility of lysosomal hydrolases*

A mitochondrial preparation was pre-incubated for 10 min. at 25° in 0·25 M-sucrose in the presence of 1:10 lecithinase, 4 mM-$CaCl_2$ and 15 mM-glyoxaline HCl buffer, pH 7·4. EDTA, to a final concentration of 8 mM, was added after pre-incubation. After removal of a sample for the measurement of total enzymic activities, the suspension was centrifuged at 3 000 000 *g*-min. in the no. 40 rotor of the Spinco Model L preparative ultracentrifuge and the supernatant was assayed similarly to determine the unsedimentable activities. The control was treated in an identical manner, except that EDTA was added at the beginning of pre-incubation.

	Enzymic activities		
		Unsedimentable (% of total)	
Hydrolase	Total (% of control)	Control	Treated
Acid phosphatase	93·2	17·8	67·7
β-Glucuronidase	91·2	21·0	86·0
Acid ribonuclease	87·4	30·1	50·7
Acid deoxyribonuclease	104·0	23·6	35·6
Cathepsin	110·0	20·4	21·6

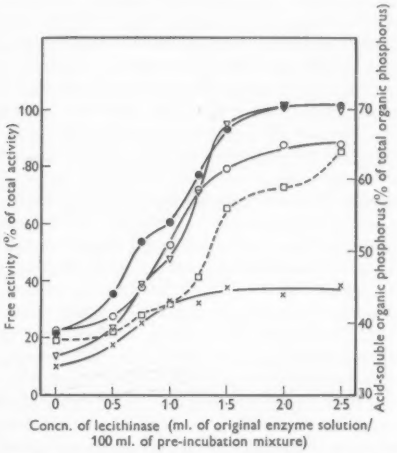

Free activity (% of total activity)
Acid-soluble organic phosphorus (% of total organic phosphorus)
Concn. of lecithinase (ml. of original enzyme solution/ 100 ml. of pre-incubation mixture)

Fig. 1. Influence of lecithinase concentration on release of acid hydrolases (left ordinate: ●, acid phosphatase; ○, β-glucuronidase; ▽, acid deoxyribonuclease; ×, cathepsin) and on appearance of acid-soluble organic phosphate (right ordinate: □). Pre-incubation for 10 min. at 25°.

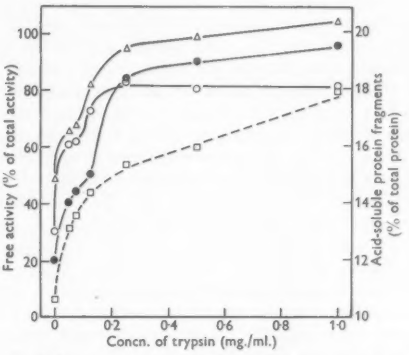


Fig. 2. Influence of trypsin concentration on release of acid hydrolases (left ordinate: ●, acid phosphatase; ○, β-glucuronidase; △, acid ribonuclease) and on appearance of acid-soluble aromatic fragments (right ordinate: □). Pre-incubation for 30 min. at 25°.

In the present experiments, an attempt was made to assess in a quantitative manner the temperature coefficients of acid phosphatase release and of endogenous proteolysis.

For the former determination, samples of a freshly prepared mitochondrial fraction were incubated at 19, 28, 37 and 45° in a medium containing 0·25 M-sucrose and 0·05 M-sodium acetate–acetic acid buffer, pH 5·0. Measurements of the free and total acid phosphatase activities were made at regular intervals. As found before, the release of acid phosphatase followed a sigmoid curve, which, however, had the same shape at all temperatures, so that the relative rates of activation could be estimated from the time necessary to reach a given arbitrary value (40 %). Endogenous proteolysis was studied by measuring the trichloroacetic acid-soluble aromatic fragments set free under the same conditions of pH and temperature in a Blendor-treated preparation.

In Fig. 3 are shown Arrhenius plots of the values found. From these plots, activation energies of 19 000 and 17 200 cal. mol.$^{-1}$ were calculated for the activation of acid phosphatase and for the autolysis of proteins respectively.

DISCUSSION

The experiments described in this paper have established that pretreatment of mitochondrial preparations with lipolytic or proteolytic enzymes, but not with ribonuclease or lysozyme, causes a

‡ Difference from controls significant at $P < 0{\cdot}001$.
† Difference from controls significant at $P < 0{\cdot}01$.
§ Difference from controls significant at $P < 0{\cdot}05$.
Values expressed as percentages of total activity before pre-incubation with trypsin.

gradual release of all lysosomal hydrolases. These phenomena are most readily interpreted as consequences of an enzymically or chemically induced disorganization of a lipoprotein barrier, restricting the physical freedom of the acid hydrolases as well as their ability to interact with their substrates. These two restrictions are obviously related, but not in an entirely rigid fashion, since the former concerns the molecules of enzyme, whereas the latter may conceivably involve only those of the substrate. Also the effectiveness of the barrier in imposing them does not necessarily depend only on its own properties, but may also be influenced by characteristics of the individual enzymes and of their respective substrates.

Such factors could account for the differences in behaviour of individual enzymes, which were occasionally encountered in the present investigations and which contrast with the close parallelism observed previously with grosser activating procedures. Considering, for instance, the effects of lecithinase, an enzyme which removes phosphorylcholine from phospholipids, it is obvious from the data of Tables 1 and 2 and of Fig. 1 that the injury inflicted on the lysosome structure is sufficient to allow a considerable part of acid phosphatase and of β-glucuronidase to diffuse into the medium and to enable the totality of these hydrolases to react freely with their respective substrates under the conditions of the free activity assays. The other extreme is represented by cathepsin, which is not solubilized at all and shows a maximal activation of 40%, whereas the nucleases are intermediate, being released into the medium to a fairly small extent, although reacting completely in the free activity measurements.

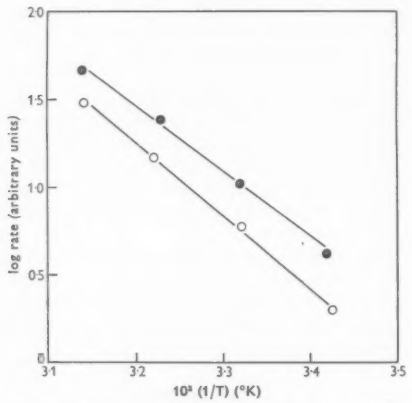


Fig. 3. Influence of temperature on rate of release of acid phosphatase (○) in 0·25 M-sucrose + 0·05 M-sodium acetate–acetic acid buffer, pH 5·0, and on rate of spontaneous proteolysis (●) in 0·05 M-sodium acetate–acetic acid buffer, pH 5·0, after disintegration in the Blendor.

As pointed out before, the two types of measurements made are vitiated to some extent by artifacts which tend to exaggerate the differences between them. Owing to adsorption phenomena, the values for physical release (solubilization) are underestimated; on the other hand, the accessibility of the enzymes (free activities) may be overestimated if further disruption of the particles occurs during the assays themselves. With acid phosphatase, which is known to be fairly strongly adsorbed by mitochondrial sediments (Berthet *et al.* 1951), solubilization may be considered as practically complete and suffices therefore to account for the activation results. This is also true of β-glucuronidase, but not of the acid nucleases, a fair proportion of which appears to remain attached to particles. They do, however, react fully in the free activity assays, either because their release is completed under the combined effects of the acidity and temperature prevailing during these assays, or because their substrates are able to penetrate freely within the particles in which they are trapped. Whichever the reason, it is obvious that all the nuclease-containing particles have been injured by the phospholipase. With cathepsin, we go one stage further; there is no detectable physical release, but a sufficient degree of permeation of the particles occurs to allow the free protease activity to reach 40% of its maximum value under the conditions of the free activity measurements. Although one cannot exclude the alternative possibility that 60% of the cathepsin-containing particles have not been damaged at all by lecithinase, it seems much more likely that all the particles have been damaged, but that a somewhat more drastic injury is necessary to allow the enzyme to diffuse freely out of the particles and possibly also to allow haemoglobin to enter them. It is also possible that less additional activation occurs in the course of the assay when haemoglobin is present in the medium.

How these observations bear upon the already frequently debated problem of the unity or plurality of the lysosomes is difficult to decide at the present time, but it is obvious that they weaken to some extent the argument put forward previously in support of the monistic hypothesis and based on the strict parallelism in the mode of release of all five lysosomal enzymes. However, sedimentation experiments have already made it clear that lysosomes form a heterogeneous population and it is therefore not particularly surprising that, as more data accumulate and new methods of activation are discovered, evidence of this heterogeneity also should appear in studies of the release of the lysosomal enzymes. Reasons for considering

the particles as forming a distinct class still remain overwhelming, but the possibility that the class may contain more than one species cannot be excluded.

With respect to thermal release, the relatively low activation energy of the process suggests that it is due to enzyme action rather than to denaturation. Cathepsin is a very good candidate for this function. As a protease, it is presumably capable of disrupting the particle membrane; it is almost inactive at pH 6·0, where the particles are relatively stable, and increases in activity up to pH 3·6, as does the lability of the particles, which are much more fragile at pH 5·0 (Appelmans & de Duve, 1955) and even more so at lower pH values (Gianetto & de Duve, 1955); finally, the activation energy of catheptic proteolysis as determined in these experiments is of the same order of magnitude as that of the release of acid phosphatase, the small difference observed being easily explained by the fact that the proteins attacked cannot have been the same in the two cases. The rapidity with which lysosomes are disrupted in acid media further suggests that if cathepsin is involved it must exert some type of selective action on the lysosomes, since these constitute only a small part of the particles present in the mitochondrial fractions studied. The possibility thus arises that the internal cathepsin of the lysosomes may actually be the agent of their thermal activation at an acid pH.

SUMMARY

1. The percentage of free activity of five lysosomal enzymes (acid phosphatase, β-glucuronidase, cathepsin, acid ribonuclease and acid deoxyribonuclease) has been measured on mitochondrial fractions from rat liver pre-incubated under mild conditions with various enzyme preparations.

2. *Clostridium welchii* lecithinase, trypsin, chymotrypsin and pancreatin all caused a more or less important release of all enzymes which could be assayed. Ribonuclease and lysozyme, on the other hand, were without effect on the state of the acid hydrolases.

3. In confirmation of previous data, these results have been taken to indicate that the release of the lysosomal enzymes is due to the disruption of a lipoprotein barrier restricting their physical freedom and their accessibility to their respective substrates.

4. Further indications have been obtained that the internal cathepsin of the particles may be involved in the spontaneous disruption of the lysosomes which occurs during incubation at pH 5·0 and 37°. On the other hand, it has been found that this enzyme lags behind the others when their release is accomplished by the action of lecithinase.

This work was supported by grants from the Centre National de Recherches sur la Croissance normale et pathologique, the Centre National de Recherches Enzymologiques, the Rockefeller Foundation and the Lilly Research Laboratories. The authors are indebted to Dr B. Cinader and to the Novo Laboratories for their generous gifts of enzymes.

REFERENCES

Allard, C., de Lamirande, G. & Cantero, A. (1955). *Biochim. biophys. Acta*, **18**, 578.
Appelmans, F. & de Duve, C. (1955). *Biochem. J.* **59**, 426.
Beaufay, H. (1957). *Arch. intern. Physiol. Biochim.* **65**, 155.
Beaufay, H. & de Duve, C. (1954). *Bull. Soc. Chim. biol., Paris*, **36**, 1551.
Berthet, J., Berthet, L., Appelmans, F. & de Duve, C. (1951). *Biochem. J.* **50**, 182.
de Duve, C. (1958). *Expos. ann. Biochim. méd.* **20**, 197.
de Duve, C. (1959). *Subcellular Particles*, p. 128. Washington: American Physiological Society.
de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955). *Biochem. J.* **60**, 604.
Finkenstaedt, J. T. (1957). *Proc. Soc. exp. Biol., N.Y.*, **95**, 302.
Gianetto, R. & de Duve, C. (1955). *Biochem. J.* **59**, 433.
Kunitz, M. (1940). *J. gen. Physiol.* **24**, 15.
Kunitz, M. & Northrop, J. H. (1935). *J. gen. Physiol.* **18**, 433.
McDonald, M. R. (1949). *J. gen. Physiol.* **32**, 39.
MacFarlane, M. G. & Datta, N. (1954). *Brit. J. exp. Path.* **35**, 191.
Paigen, K. & Griffiths, S. K. (1959). *J. biol. Chem.* **234**, 299.
Rademaker, W. & Soons, J. B. J. (1957). *Biochim. biophys. Acta*, **24**, 451.
Rotherham, J., Schottelius, D. D., Irvin, J. L. & Irvin, E. M. (1956). *J. biol. Chem.* **223**, 817.
Roy, A. B. (1958). *Biochem. J.* **68**, 519.
Sellinger, O. & Doyen, A. (1959). *Arch. int. Physiol. Biochim.* **67**, 519.
Shack, J. (1957). *J. biol. Chem.* **226**, 573.
Viala, R. & Gianetto, R. (1955). *Canad. J. Biochem. Physiol.* **33**, 839.
Wattiaux, R., Baudhuin, P., Berleur, A. & de Duve, C. (1956). *Biochem. J.* **63**, 608.
Wattiaux, R. & de Duve, C. (1956). *Biochem. J.* **63**, 606.

Tissue Fractionation Studies

10. INFLUENCE OF ISCHAEMIA ON THE STATE OF SOME BOUND ENZYMES IN RAT LIVER

By C. de DUVE and H. BEAUFAY*
Department of Physiological Chemistry, University of Louvain, Belgium

(*Received* 24 *March* 1959)

Cathepsin and other intracellular acid hydrolases have long been suspected of playing an important role in the aseptic autolysis and necrosis of tissues, but no satisfactory explanation has yet been given of their relative lack of activity in intact cells, nor of the processes responsible for their activation in dying or dead cells (see, for instance, the review by Bradley, 1938). As pointed out before (de Duve & Berthet, 1954), the finding that these enzymes are segregated within particles which are impermeable to their respective substrates (lysosomes; see de Duve, Pressman, Gianetto, Wattiaux & Appelmans 1955) provides an obvious answer to the first question. This, however, implies as a necessary corollary and answer to the second question that the rupture of the particles *in situ* and consequent release of their enzymic content must be an early step in the chain of processes leading to the autolytic breakdown of tissue constituents.

The experiments described in this paper were designed to test this hypothesis on a simple and reproducible material, namely on liver tissue entirely deprived of blood supply. Rat liver proved particularly suitable for this kind of study, since the hilum of one lobe can easily be ligated without injuring the remainder of the tissue, which may thus serve as an internal control. The results obtained indicate that lysosomes do indeed suffer a rapid disintegration or increase in fragility in necrotizing liver. The results have been previously reported briefly (Beaufay & de Duve, 1957).

METHODS

Adult rats of either sex were laparotomized under Nembutal anaesthesia. The vascular pedicle of the left liver lobe was ligated firmly with fine thread, care being taken not to injure the adjacent part of the liver, and the abdominal cavity was closed. This operation was well tolerated by the animals, which started moving and feeding normally as soon as they had recovered from the anaesthetic. They were kept in a warm enclosure and killed at times between 0·5 and 48 hr. after the ligature had been placed. The ligated lobe was removed and the remainder of the liver was trimmed of all parts which appeared to have suffered from the operation. This included the left middle lobe, since a collateral vessel irrigating this lobe was frequently enclosed in the ligature.

The two liver fragments were cooled and weighed in ice-cold 0·25 M-sucrose, and then homogenized in the same medium by means of a smooth-glass tube fitted with a polytetrafluoroethylene (Teflon) pestle (manufactured by A. H. Thomas Co., Philadelphia, Pa., U.S.A.). This was done according to a rigorously standardized grinding technique consisting of three complete up-and-down runs of the pestle rotating at about 1000 rev./min. The tubes were kept in a beaker of crushed ice during the whole operation and contained the liver samples in 15 ml. of 0·25 M-sucrose. The homogenized suspensions were subsequently brought to a volume (ml.) corresponding to 10 times the weight (g.) of tissue processed. Part of the homogenates was kept for analysis and the remainder spun for 30 min. at 40 000 rev./min. (3 000 000 *g* min. in the middle of the fluid) in the no. 40 rotor ($r_{max.}$ = 8·1 cm., $r_{min.}$ = 4·8 cm.) of the Spinco model L preparative ultracentrifuge.

The homogenates were analysed for nitrogen by a micro-Kjeldahl method, for cytochrome oxidase and glucose 6-phosphatase according to de Duve *et al.* (1955), and for total acid phosphatase, β-glucuronidase, cathepsin, acid ribonuclease and acid deoxyribonuclease in the presence of 0·1% of Triton X-100 (Rohm and Haas Co., Philadelphia, Pa., U.S.A.), as described by Wattiaux & de Duve (1956). Free activities of lysosomal hydrolases were also measured in some cases according to de Duve *et al.* (1955), but these determinations were complicated by high and rapidly changing blanks. For this reason, the degree of binding of the lysosomal hydrolases was assessed by measuring their total activity in the high-speed supernatants of the homogenates.

In the experiments on liver slices, the slices were prepared as described by Vuylsteke & de Duve (1957), placed in stoppered flasks containing the medium and the chosen gas mixture and shaken at 37° in a Warburg bath. After incubation, they were transferred into a homogenizer and treated as described above for the liver samples. The substrate mixtures used to measure the enzymic activities contained all the components of the incubation medium, to prevent an osmotic disruption of the particles in the free activity assays. The experiments on isolated particles were conducted in a similar manner on mitochondrial fractions prepared according to Appelmans & de Duve (1955).

* Chargé de recherches du FNRS.

RESULTS

General findings

Altogether thirty-three animals were operated on and killed, in groups of three, 0·5, 1, 2, 3, 4, 6, 9, 12, 18, 24 and 48 hr. after operation. The ischaemic lobe was seen to darken rapidly after ligation and later became spotted on its surface with haemorrhagic flecks. After a few hours discoloured areas began to appear and to spread out, becoming confluent at the end of 12 hr. At this time the lobe had acquired a uniform greyish colour and a gummy consistency, which persisted without appreciable further change for the next 36 hr. Immediate swelling occurred only if the ligature had not been tight enough, in which case the animal was discarded. A slow progressive swelling developed during the first 8–12 hr. after ligation, presumably owing to imbibition of water from the peritoneal cavity. This was exemplified by a greater resilience of the ligated lobe, a significant increase in the ratio of the total liver weight (ligated + unligated) to the weight of the animal (Table 1) and a lowering of the nitrogen content per unit weight of the ischaemic part with respect to that of the remainder of the liver (Fig. 1). The last phenomenon could be due also to losses of nitrogenous material by diffusion, but, in view of the other evidence, was attributed mainly to gain of water. It was accordingly decided to relate the results of the enzymic determinations to the nitrogen content rather than to the weight of tissue.

Table 1. *Influence of ischaemia on total weight of liver*

Results were obtained 6 hr. or more after ligation. For operated animals, weight = sum of weights of ligated and unligated fragments. Unoperated animals are control group of another experiment performed at the same time.

Group	No. of animals	Mean relative wt. of liver ± S.E.M. (g./100 g. animal)
Unoperated	19	4·23 ± 0·11
Operated	16	4·92 ± 0·16
Difference	—	0·69 ± 0·19
		$P = 0·001$

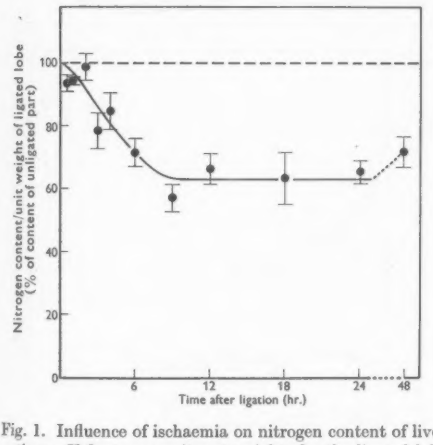


Fig. 1. Influence of ischaemia on nitrogen content of liver tissue. Values, per unit wet weight, for the ligated lobe are given as percentages of corresponding values for the unligated part. Means of three animals, with S.E.M., are given.

Enzymic determinations

In a number of control experiments, it was found that the enzymic activities measured were all identical in the two parts of the liver and that the unligated portion therefore provided a valid internal control for the ligated lobe. In addition, no significant changes were observed in the total activities of the unligated part as a result of the operation. For these reasons, the values (in units/mg. of nitrogen) found for a ligated lobe have simply been expressed as percentages of the corresponding values for the unligated part of the same liver.

In Figs. 2 and 3 are shown the results obtained for cytochrome oxidase and glucose 6-phosphatase. After ligation, both enzymes suffer a progressive inactivation to about one-tenth of their activity in the control fragment. This value is reached in about 6–8 hr. for cytochrome oxidase and 10–12 hr. for glucose 6-phosphatase, and is then maintained almost unchanged, even after 48 hr.

The data gathered on the five lysosomal enzymes are represented graphically in Figs. 4–8. For the total activities, three types of behaviour are seen. Acid phosphatase shows no change for the first 24 hr. and then decreases to half its normal value during the second day after ligature. Cathepsin and the two acid nucleases show a fairly rapid initial decrease, reaching 50–60% of the control values after about 8 hr. (somewhat earlier with ribonuclease) and followed by an almost imperceptible secondary fall. Finally, β-glucuronidase remains unchanged for the first 6 hr., then increases by about 40% during the next 12 hr. and remains elevated up to 48 hr. after ligation.

The unsedimentable activities of the lysosomal hydrolases show early changes which are the same for all five enzymes. In the unligated control fragment, they exhibit a slight rise during the first hours after operation and subsequently fall back to their normal values. A much more pronounced increase of these activities occurs in the ligated lobe, reaching a plateau in about 3–4 hr. With

β-glucuronidase, this increase is followed after 6 hr. by a large secondary rise coinciding with the enhancement of the total activity.

In the figures just described, the unsedimentable activities are expressed as percentages of the total activities of the unligated part. Since these do not change as a result of the operation, the curves drawn show the changes suffered by the absolute values of the unsedimentable activities. The actual state of the lysosomes is probably more correctly appreciated by relating the unsedimentable activities to the corresponding total activities. This has been done in Fig. 9, in which the relative values for acid phosphatase, cathepsin and the two nucleases have been further averaged, to provide a condensed

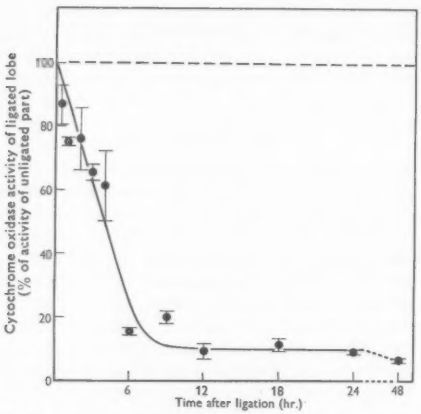


Fig. 2. Influence of ischaemia on cytochrome oxidase activity of liver tissue. Values, in units/mg. of N, for the ligated lobe are given as percentages of corresponding values for the unligated part. Means of three animals, with S.E.M., are given.

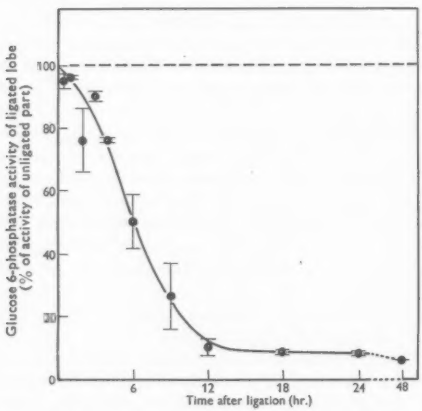


Fig. 3. Influence of ischaemia on glucose 6-phosphatase activity of liver tissue. Values, in units/mg. of N, for the ligated lobe are given as percentages of corresponding values for the unligated part. Means of three animals, with S.E.M., are given.

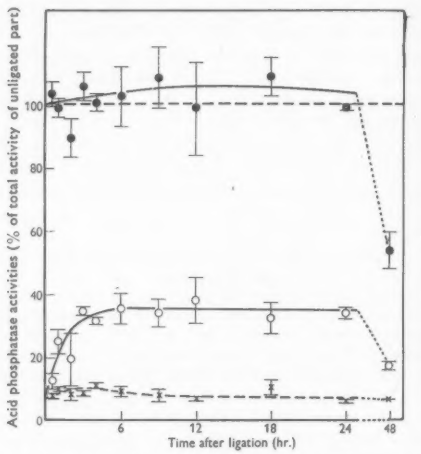


Fig. 4. Influence of ischaemia on acid phosphatase activity of liver tissue. Ligated lobe: ●, total activity; ○, unsedimentable activity. Unligated part: ×, unsedimentable activity. All values, in units/mg. of N, are given as percentages of total activity of the unligated part. Means of three animals, with S.E.M., are given.

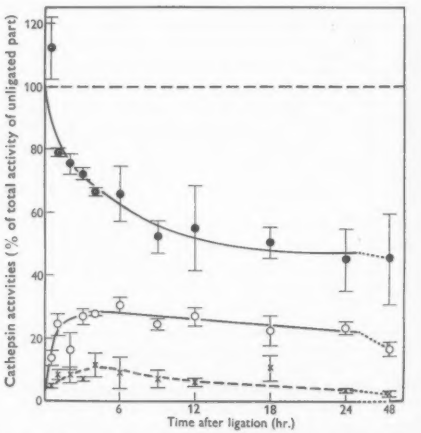


Fig. 5. Influence of ischaemia on cathepsin activity of liver tissue. For explanation of symbols see Fig. 4.

view of the results obtained. The values for β-glucuronidase, which behaves atypically after 6 hr., have been left out of this composite picture.

To clarify the significance of the plateau shown by the upper curve in Fig. 9, free acid phosphatase was measured in addition to the total and unsedimentable activities of this enzyme, on normal livers and on ligated and unligated lobes extirpated 2 and 16 hr. after ligation. The results of these assays are given in Table 2. They show that the release of acid phosphatase, though very extensive, is probably not complete even long after the phenomenon has clearly come to an end. Apparently, some 20 % of the particles containing this enzyme escape destruction under the conditions of our experiments. It will also be noted that the ratio of the free to the unsedimentable activity decreases as more enzyme is released. This is understandable since there will be fewer intact granules capable of contributing to the free activity, and the proportion of adsorbed enzyme will tend to decrease.

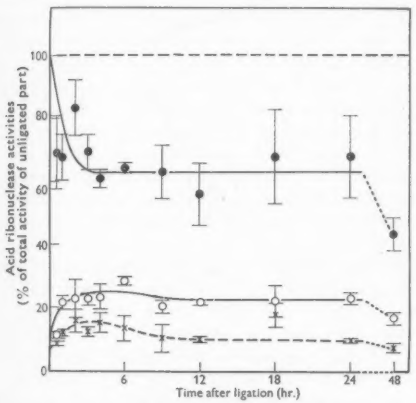


Fig. 6. Influence of ischaemia on acid ribonuclease activity of liver tissue. For explanation of symbols see Fig. 4.

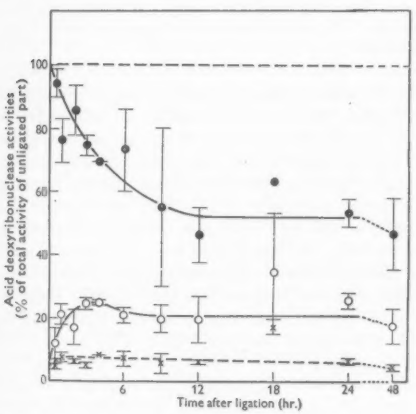


Fig. 7. Influence of ischaemia on acid deoxyribonuclease activity of liver tissue. For explanation of symbols see Fig. 4.

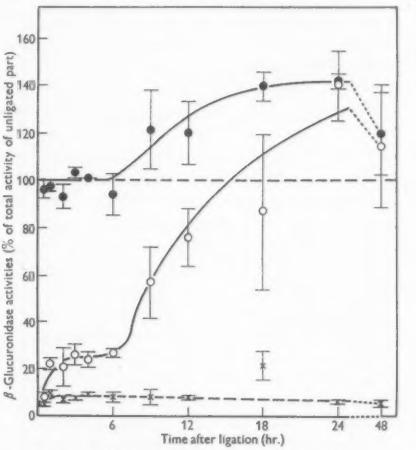


Fig. 8. Influence of ischaemia on β-glucuronidase activity of liver tissue. For explanation of symbols see Fig. 4.

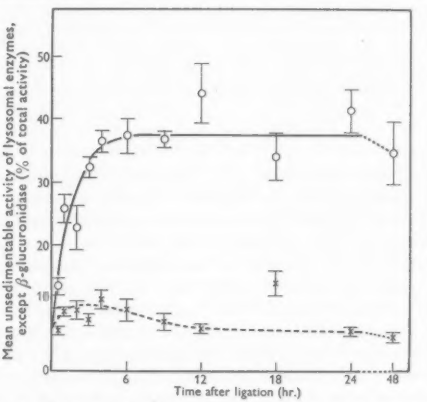


Fig. 9. Influence of ischaemia on partition of lysosomal hydrolases between particulate and soluble phases. Mean unsedimentable activities, with S.E.M., are given for acid phosphatase, cathepsin, acid ribonuclease and acid deoxyribonuclease, expressed as percentages of corresponding total activities: ○, ligated lobe; ×, unligated part.

Table 2. *Comparison of free and unsedimentable acid phosphatase activities in ligated and unligated tissue*

Tissue	No. of expts.	Percentage of total acid phosphatase (mean ± S.E.M.) Free activity	Unsedimentable activity	Ratio (F/U)
Normal	4	20·9 ± 1·0	7·4 ± 0·8	2·82
Unligated lobe from operated animals	8	20·1 ± 0·8	8·4 ± 0·2	2·39
Ligated lobe (2 hr.)	3	47·4 ± 1·7	25·0 ± 0·6	1·90
Ligated lobe (16 hr.)	8	78·7 ± 2·3	42·6 ± 1·2	1·85

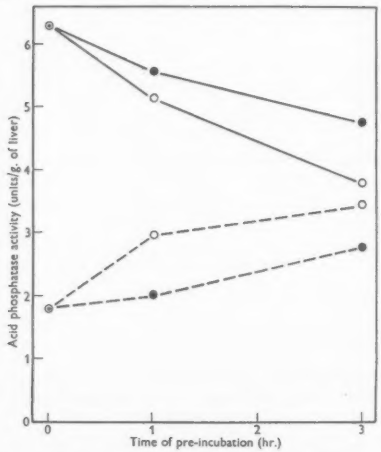


Fig. 10. Influence of anoxia on acid phosphatase in liver slices. Rat-liver slices were incubated at 37° under constant shaking in a medium of pH 7·6, containing 0·11 M-KCl and 0·04 M-$KHCO_3$, equilibrated with 5% of CO_2 in O_2 (●) or in N_2 (○). Continuous lines show total activity, and broken lines free activity, of enzyme in tissue homogenized at times indicated in the abscissa.

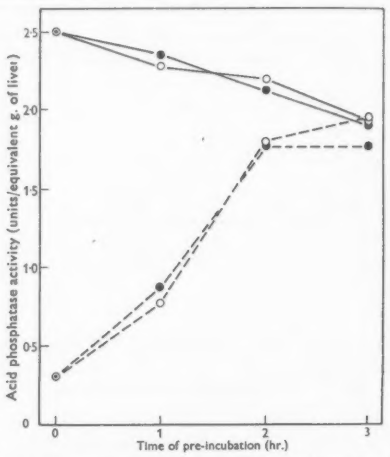


Fig. 11. Influence of anoxia on acid phosphatase in mitochondrial fraction. Particles were incubated at 37° under constant shaking in a medium of pH 7·1, containing 0·25 M-sucrose, 0·175 M-KCl and 0·0125 M-$KHCO_3$, equilibrated with 5% of CO_2 in O_2 (●) or in N_2 (○). Continuous lines show total activity, and broken lines free activity, of enzyme measured at times indicated in the abscissa.

Influence of anoxia

Anoxia represents one of the main consequences of ischaemia and it seemed of interest to find out whether oxygen deprivation alone could cause the release of the lysosomal enzymes. To test this possibility, a number of experiments were performed on rat-liver slices and on mitochondrial fractions, incubated at 37° under constant shaking in a bicarbonate medium, equilibrated with a gas phase containing 5% of CO_2 in either O_2 or N_2. The state of the lysosomes was followed at different intervals by measuring the free and total acid phosphatase activities on homogenates prepared from the slices or on the isolated particles. These experiments were complicated by losses of acid phosphatase, which, when in soluble form, was found to be unstable at 37° and at pH values higher than 6·0. It was also observed that even well-oxygenated slices incubated under conditions which have been found suitable for the study of several metabolic processes show a rapid deterioration of their lysosomes, exemplified by the appearance of acid phosphatase in the incubation medium and by an increase in the free activity of this enzyme in the homogenized tissue. However, as shown in Fig. 10, the release and subsequent loss of acid phosphatase is significantly greater when the slices have been incubated anaerobically. Isolated particles, on the other hand, are not influenced by the availability of oxygen (Fig. 11).

DISCUSSION

The results reported in this paper show that complete ischaemia of the liver modifies the partition of lysosomal hydrolases between the particulate and

soluble phases of the homogenized tissue. As shown most clearly by Fig. 9, the average proportion of activity found in the high-speed supernatants increases from an initial value of less than 10% to a plateau value of about 40%. The proportion of injured lysosomes corresponding to these figures is undoubtedly much greater, since the released enzymes are partly carried down with the sediment owing to adsorption and other trapping phenomena. From the acid phosphatase values given in Table 2 it may be estimated that about 20% of the lysosomal enzymes occur in free form in homogenates of normal tissue and that this proportion reaches 80% in homogenates of tissue maintained ischaemic for 4 hr. or more. Apparently, a small fraction of the lysosomes escape injury, even after prolonged ischaemia. Since about one-tenth of cytochrome oxidase and glucose 6-phosphatase is also spared, it is possible that some of the liver cells survive under the conditions of these experiments.

As compared with other biochemical changes observed in this and other similar investigations, the phenomenon involved in the release of the lysosomal hydrolases appears to be a fairly rapid one, since it comes to an end at a time when the cells still retain more than 60% of their cytochrome oxidase and 75% of their glucose 6-phosphatase activities, and when other morphological and chemical signs of autolysis are still very discreet (Berenbom, Chang, Betz & Stowell, 1955).

The nature of the intracellular change responsible for the lysosomal alterations observed on the homogenates is, of course, not known. It could be a true rupture of the lysosomes, with consequent release of their enzymes within the cells, or an increase in fragility of the particles, causing them to break up more easily upon homogenization. It could even have nothing to do with the particles themselves and consist, for instance, of the formation in other parts of the cells of a lytic substance which causes the rupture of the particles when brought together with them by the homogenization procedure.

With regard to these various hypotheses, it should be pointed out that, with the exception of β-glucuronidase, which will be discussed below, the lysosomal enzymes undergo no further change in partition between 4 and 48 hr. of observation. It seems improbable that the lysosomes are still intact after 2 days of ischaemia, when the tissue is in an advanced state of autolysis, since this would imply not only that the acid hydrolases have actually nothing to do with the autolytic process, but even that the particles in which they are contained are relatively refractory to this process. If therefore we take the homogenate picture after 48 hr. as a fairly faithful representation of the intracellular state, the point at issue becomes whether the intracellular lysosomal rupture does in fact follow the time course indicated by the results on homogenates or whether it is slower. In other words, how near spontaneous rupture must the average lysosome be to break up during homogenization? Whichever the answer to this question, it is clear that the process which brings the particles to the critical stage of fragility is both rapid and early, and it seems therefore reasonable to assume that the intracellular rupture is not too far removed from the point of breakage *in vitro*.

Thus as much by their nature as by their time course and magnitude, the observed lysosomal alterations bring direct support to the hypothesis, outlined in the introduction, that the release of lysosomal enzymes occurs as an early step in autolysis.

Our results also shed some light on the possible mechanism whereby the lysosomes become ruptured. We find that anoxia alone may precipitate the phenomenon in slices, but not in isolated particles. The latter detail is of importance since it suggests that the lysosomes are sensitive, not to the oxygen partial pressure itself, but to some environmental change taking place in anoxic cells. As a possible link in the chain of events one could think of the lowering of the pH, which is known to accelerate considerably the breakdown of lysosomes *in vitro*, by a mechanism which, as shown in the preceding paper (Beaufay & de Duve, 1959), may involve the particles' own cathepsin acting on the membrane from the inside. The properties of this enzyme are compatible with such a role, since it is known to be practically inactive above pH 6·0 and to increase rapidly in activity as the pH falls below this limit.

Whichever the mechanism involved, the data obtained suggest that the autolysis of anoxic cells may start at the site of the lysosomes, possibly with a primary attack of the lysosomal membrane, and spreads out from these centres as the released hydrolases diffuse outwards. Such a process could conceivably play a causal role in cell death, but it could also occur as a consequence of this phenomenon which may itself be due to more rapid alterations, unrelated to the invasion by lysosomal enzymes. Further investigations on the reversibility of the observed changes may possibly help to answer this question. Of interest in this respect is the slight increase in the unsedimentable fraction of lysosomal enzymes which takes place in the control tissue during the first hours after operation, possibly as a consequence of the operative shock. Notable also is the rapid deterioration of lysosomes in isolated liver slices, even under aerobic conditions. The inner layers of cells in such slices are known to be largely deprived of oxygen and the phenomenon is therefore not surprising, but it may

help to explain some of the failures to demonstrate hormonal actions on such systems, in particular with insulin.

In addition to the changes already discussed, our experiments have revealed various alterations in the activity of the enzymes themselves. Most clear-cut is the inactivation of the mitochondrial cytochrome oxidase and of the microsomal glucose 6-phosphatase, which presumably reflects a pathologically accelerated degradation, since a very high turnover would have to be assumed for these enzymes, were their decay simply due to a cessation of synthesis. The partial inactivation suffered by cathepsin and by the nucleases is more puzzling, since it starts fairly rapidly but slows down considerably at a later stage. Finally, the behaviour of β-glucuronidase is particularly striking, since this enzyme undergoes an extensive secondary solubilization, accompanied by a 40% increase in total activity and starting rather suddenly about 6 hr. after ligation. It may be recalled that, unlike the other acid hydrolases of the group, β-glucuronidase is not exclusively located in the lysosomes but also occurs to a significant extent in the microsomes, where it shows a different pH–activity curve (de Duve *et al.* 1955). It is tempting to relate the secondary rise in total and soluble activity of this enzyme to an autolytic release of the microsomal component. It may be finally pointed out that all five lysosomal hydrolases are still present at half or more of their initial concentration 48 hr. after the blood supply has been cut off. As such, they show a length of survival compatible with their assumed role in the process of autolysis.

SUMMARY

1. The activity and state of a number of bound enzymes have been followed in a rat-liver lobe rendered completely ischaemic by ligation of the vascular pedicle. The unligated part of the same liver was used as an internal control. The enzymic determinations were performed on the homogenized tissue samples and on high-speed supernatants from these homogenates.

2. The earliest change observed after complete ischaemia was a progressive release of the lysosomal hydrolases, acid phosphatase, β-glucuronidase, cathepsin, acid ribonuclease and acid deoxyribonuclease. This release reached a plateau, corresponding to the rupture of about 80% of the lysosomes, in the space of 3–4 hr. Whether the phenomenon actually occurred within the cells or only upon homogenization is not known, but the former interpretation is favoured as providing a satisfactory explanation of how autolytic processes are initiated in necrotizing cells.

3. Changes in total enzymic activities were as follows: cytochrome oxidase lost about nine-tenths of its activity in 6–8 hr. and then remained constant; glucose 6-phosphatase did the same in 10–12 hr.; acid phosphatase remained unchanged during the first day and then slowly decreased to about half its value during the second day; cathepsin and the two acid nucleases suffered a fairly rapid partial inactivation, reaching 50–60% of their activity after about 8 hr., and then decreased very slowly; β-glucuronidase was unaffected for 6 hr. and then increased by about 40% during the next 12 hr. and remained elevated. All five lysosomal hydrolases were still present at half or more their initial concentration 48 hr. after the blood supply had been cut off, thus showing a survival time consistent with their assumed role in autolysis.

4. From studies on isolated liver slices and on particles it appears that the ischaemic release of lysosomal hydrolases may be due to an anoxic change in the intracellular environment of the particles, possibly a lowering of the pH.

This work was supported by grants from the Centre National de Recherches sur la Croissance normale et pathologique, the Centre National de Recherches Enzymologiques, the Rockefeller Foundation and the Lilly Research Laboratories.

REFERENCES

Appelmans, F. & de Duve, C. (1955). *Biochem. J.* **59**, 426.
Beaufay, H. & de Duve, C. (1957). *Arch. intern. Physiol. Biochem.* **65**, 156.
Beaufay, H. & de Duve, C. (1959). *Biochem. J.* **73**, 604.
Berenbom, M., Chang, P. I., Betz, H. E. & Stowell, R. E. (1955). *Cancer Res.* **15**, 1.
Bradley, H. C. (1938). *Physiol. Rev.* **18**, 173.
de Duve, C. & Berthet, J. (1954). *Int. Rev. Cytol.* **3**, 225.
de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955). *Biochem. J.* **60**, 604.
Vuylsteke, C. A. & de Duve, C. (1957). *Arch. int. Pharmacodyn.* **111**, 437.
Wattiaux, R. & de Duve, C. (1956). *Biochem. J.* **63**, 606.

Tissue Fractionation Studies

11. INFLUENCE OF VARIOUS HEPATOTOXIC TREATMENTS ON THE STATE OF SOME BOUND ENZYMES IN RAT LIVER

By H. BEAUFAY,* E. van CAMPENHOUT and C. de DUVE
Departments of Physiological Chemistry and of Microscopic Anatomy, University of Louvain, Belgium

(*Received* 24 *March* 1959)

The studies described in the preceding paper (de Duve & Beaufay, 1959) having revealed, amongst other enzymic alterations, a rapid release of lysosomal enzymes in ischaemic liver, it appeared of interest to perform similar determinations on animals subjected to various other treatments reputed to cause liver damage. The results of these investigations are reported in this paper, together with similar data obtained on a few animals treated with hydrocortisone. The results of an extensive investigation of the same kind performed on animals fed with carcinogenic dyes have been published elsewhere (Deckers-Passau, Maisin & de Duve, 1957).

METHODS

The experiments were performed on rats of either sex, weighing from 100 to 150 g. The animals were divided into groups A–G.

(A) Controls, fed *ad lib.* a standard diet containing 45% of ground wheat, 40% of ground oats, 8% of fish meal, 3% of skimmed milk, 2% of cod-liver oil, 1% of dry yeast and 1% of salt.

(B) Animals fasted for 6 days but supplied with plenty of drinking water.

(C) Animals fasted as in group B, and then given access to food 8 hr. before being killed.

(D) Animals on diet A, treated for 5 days with a daily subcutaneous injection of 0·2 ml. of carbon tetrachloride in 0·8 ml. of olive oil, and killed on the following day.

(E) Animals receiving 8 g. daily of a necrogenic diet (containing 73% of ground maize, 18% of dry bakers' yeast, 5% of arachis oil, 1% of cod-liver oil and 3% of salt) and supplemented with a daily dose of 20 μg. of thiamine hydrochloride, 25 μg. of riboflavin, 20 μg. of pyridoxine and 100 μg. of calcium pantothenate (Glynn & Himsworth, 1944).

(F) One animal on diet A, killed 8 days after ligation of the main bile duct.

(G) Animals on diet A, treated for 3 days with a daily subcutaneous injection of 12·5 mg. of hydrocortisone and killed on the following day.

In addition, a more limited experiment was performed on two groups of animals subjected to a prolonged period of starvation (6–8 days) without preliminary treatment (H) or after intoxication with carbon tetrachloride as in group D (J).

As a rule, three animals belonging to three different groups and one control were killed on the same day. Their livers were homogenized, centrifuged and analysed as described in the preceding paper (de Duve & Beaufay, 1959). Samples of liver were also fixed in Bouin's fluid for histological examination.

This design made it possible to assign to each group a set of comparable control animals and to take into account eventual variations due to seasonal effects or other uncontrolled factors. However, subsequent statistical analysis revealed no significant trend amongst the control animals and these were therefore treated as a single group, against which all others were compared.

RESULTS

In Table 1 are summarized the results of the analyses performed on the animals in groups A–G. Total enzymic activities are expressed in units/g. of liver nitrogen and can be related to the wet weight of the tissue or to the weight of the animal by means of the corresponding data on liver nitrogen. The unsedimentable activities of the lysosomal hydrolases, on the other hand, are given as percentages of the corresponding total activities, a mode of expression which provides a direct estimate of the state of the lysosomes in the preparations. To assist in the perusal of the table, all values differing significantly from the control values are indicated by suitable signs.

As shown by the headings of Table 1, not all animals resisted the treatment to which they were subjected. Some were found dead and were discarded; others were killed in a comatose or precomatose state. This fact turned out to be of great interest, since typical biochemical signs were found to be associated with coma. Consequently, the results obtained on the comatose and precomatose animals will be examined separately, together with those of groups H and J.

Results on non-comatose animals

Starvation (*groups B*1 *and C*1). Except for a certain degree of shrinking of the cells, there were no consistent histological changes in the livers of the starved animals. After refeeding, however, the cells appeared swollen and vacuolated in several

* Chargé de recherches du FNRS.

Table 1. *Summary of biochemical results*

All values are means±S.E.M. Injections (D and G) were given subcutaneously.

Group	A	B				C		
Treatment	None	Complete starvation				Complete starvation+subsequent meal 8 hr. before killing		
Days of treatment	—	6				6		
Subgroup	—	1	2	3	4	1	2	3
State of animals	Normal	Non-comatose	Pre-comatose	Comatose	Dead	Non-comatose	Pre-comatose	Dead
No. of animals	20	8	1	3	1	8	1	1
Liver								
Weight (g./100 g. of animal)	4·13±0·13	3·37±0·10***	3·04	3·04±0·39*	—	3·96±0·10†††	2·81	—
Nitrogen (mg./g. of liver)	32·0±0·50	32·5±1·5	23·1	32·0±2·7	—	29·2±1·0*	30·9	—
Nitrogen (mg./100 g. of animal)	130±4·2	107±7·4*	70·0	95·0±3·9***	—	113±4·7*	87	—
Total enzymic activities (units‡/g. of N)								
Cytochrome oxidase	616±26	541±46	—	240±72***	—	496±69	256	—
Glucose 6-phosphatase	627±33	719±27*	—	398±114	—	732±69	521	—
Acid phosphatase	189±6·0	199±9·0	386	339±17***	—	174±6·0 †	301	—
β-Glucuronidase§	18·5±1·6	20·2±1·5	34·9	38·9±4·3***	—	16·8±1·3	65·0	—
Acid ribonuclease	68·3±4·3	71·1±3·0	115·5	101±25	—	62·6±5·1	78·6	—
Acid deoxyribonuclease	20·0±1·5	21·9±2·6	58·9	40·8±7·5*	—	19·7±2·2	35·3	—
Cathepsin	38·0±1·3	46·5±2·6**	64·9	85·0±5·8***	—	34·6±2·5††	78·6	—
Unsedimentable activities (% of total)								
Acid phosphatase	7·18±0·18	10·9±0·71***	16·3	32·3±3·3***	—	8·80±0·60*	40·3	—
β-Glucuronidase	5·78±0·53	8·96±0·47***	25·0	46·0±3·8***	—	7·80±0·30**	27·3	—
Acid ribonuclease	9·74±0·69	15·5±1·6**	28·0	24·8±4·4**	—	13·8±0·55***	33·3	—
Acid deoxyribonuclease	2·82±0·23	3·80±1·3	24·0	17·2±2·6***	—	6·20±0·54***	11·0	—
Cathepsin	4·94±0·32	8·58±1·0**	16·5	19·3±1·3***	—	8·10±0·57***	15·4	—

Group	D	E					F	G
Treatment	0·2 ml. of CCl_4 injected daily	Deficient diet					Ligation of main bile duct	12·5 mg. of hydrocortisone injected daily
Days of treatment	5	53±7·2	33	46±6	68	64±7	8	3
Subgroup	—	1	2	3	4	5	—	—
State of animals	Non-comatose	Non-comatose	Comatose, gastr. perf.	Comatose	Comatose	Dead	Non-comatose	Non-comatose
No. of animals	9	7	1	3	1	7	1	4
Liver								
Weight (g./100 g. of animal)	6·36±0·41***	4·12±0·20	4·25	2·79±0·28***	3·93	—	3·67	6·15±0·25**
Nitrogen (mg./g. of liver)	24·2±0·84***	27·3±1·2**	29·5	27·6±0·70***	30·8	—	42·1	27·7±0·59**
Nitrogen (mg./100 g. of animal)	149±10·5	112±4·5**	125	77·0±7·3***	121	—	155	170±10**
Total enzymic activities (units‡/g. of N)								
Cytochrome oxidase	552±47	604±44	411	332±39***	584	—	501	369±14***
Glucose 6-phosphatase	227±26***	453±57*	390	641±28	438	—	262	660±49
Acid phosphatase	204±8·0	238±13**	321	345±28***	254	—	194	158±11*
β-Glucuronidase	17·9±2·5	14·9±1·4	23·9	17·4±3·3	17·6	—	14·9	14·4±1·4
Acid ribonuclease	84·2±9·7	74·7±5·0	136	141±38	54·9	—	100	52·9±5·3*
Acid deoxyribonuclease	26·1±3·7	38·2±2·5***	45·0	70·7±18**	25·3	—	56·2	17·9±1·3
Cathepsin	73·8±5·0***	59·8±6·6**	85·0	75·0±4·1***	54·2	—	67·1	30·9±0·54
Unsedimentable activities (% of total)								
Acid phosphatase	9·88±0·61***	9·12±0·63**	22·2	26·1±6·7*	9·70	—	9·84	7·43±0·50
β-Glucuronidase	14·9±1·6***	9·98±1·3**	29·5	41·4±1·3***	11·2	—	30·2	5·93±0·61
Acid ribonuclease	17·4±1·5***	17·5±2·5**	21·1	23·7±8·2	18·4	—	20·3	7·20±1·1*
Acid deoxyribonuclease	7·65±0·75***	7·65±0·75***	9·8	15·5±6·0	8·20	—	8·4	0·80±0·30**
Cathepsin	9·28±0·74***	9·17±1·2**	16·8	26·0±2·0***	4·90	—	13·8	3·78±0·58

* Difference from control values (A) significant at $P<0·05$.
** Difference from control values (A) significant at $P<0·01$.
*** Difference from control values (A) significant at $P<0·001$.
‡ Units are those defined by de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955).
§ Uncorrected for inhibition by sucrose (approx. 28%).
† Difference from B1 significant at $P<0·05$.
†† Difference from B1 significant at $P<0·01$.
††† Difference from B1 significant at $P<0·001$.

though not all organs. The loss in liver weight and nitrogen was proportionately greater than the total loss of weight of the animal. Refeeding caused a considerable gain in liver weight and a smaller rise in liver nitrogen. The latter change is not significant when related to the weight of the animal, but is clearly shown by the decrease in the specific activity of most enzymes.

Cytochrome oxidase appeared to be somewhat more affected by starvation than the total proteins, whereas glucose 6-phosphatase was relatively spared. Lysosomal hydrolases tended to lag behind the average proteins, with the result that their specific activity increased slightly during starvation and decreased upon refeeding. These changes were statistically significant only for cathepsin. The unsedimentable activities of the acid hydrolases were significantly increased in both groups.

Carbon tetrachloride poisoning (*group D*). Despite the high doses of carbon tetrachloride administered, the animals appeared to remain in an excellent state of health. As shown by the results of Table 2, they were even more resistant to starvation than untreated animals. Their livers were considerably enlarged, owing mostly to the accumulation of fat, and showed extensive centrilobular fatty degeneration.

Most notable amongst the enzymic modifications observed in this group were the considerable dedecrease in glucose 6-phosphatase and increase in total cathepsin, as well as the moderate rise in the unsedimentable fraction of the lysosomal hydrolases.

Deficient diet (*group E* 1). The livers of the seven animals of the group which were killed in apparent health did not differ morphologically from those of the control group except in one case where a few zones of swollen or lysed cells were seen. The liver weight was normal, but its nitrogen content was low. This relative loss of protein was paralleled by cytochrome oxidase and exceeded by glucose 6-phosphatase and possibly also β-glucuronidase, though in the latter case the change was not significant. On the other hand, acid phosphatase and ribonuclease were essentially preserved, with a consequent moderate rise in specific activity, and cathepsin and especially acid deoxyribonuclease were distinctly increased. The unsedimentable activities of all lysosomal hydrolases were significantly augmented.

Bile-duct ligation (*group F*). This animal showed typical signs of obstructive jaundice, but tolerated this condition with no outward sign of ill-being. Histologically, the liver showed a remarkable multiplication of the bile canaliculi, which were also greatly distended. The biochemical state of the liver was characterized by a high nitrogen content, a very low glucose 6-phosphatase activity and high levels of ribonuclease, cathepsin and especially deoxyribonuclease. In terms of animal weight this last enzyme reached almost four times its normal level. The unsedimentable activities of lysosomal hydrolases were all distinctly elevated. Although such results obtained on a single animal furnish only little information, it should be pointed out that several of the observed differences exceed by far the standard deviation of the normal population and are probably significant.

Hydrocortisone treatment (*group G*). The livers of these animals were enlarged considerably and showed the typical pale colour associated with a high glycogen content. The cells were hypertrophied and appeared vacuolated, presumably by glycogen deposits. The latter could not, however, be revealed histochemically, owing to improper fixation. The nitrogen content was decreased, but not in proportion to the increase in liver weight, so that the total liver nitrogen showed a 30% increase, when related to the animal weight. Glucose 6-phosphatase was the only enzyme to have participated in this hypertrophy, whereas lysosomal hydrolases appeared to exhibit a corresponding dilution, which, however, was significant only for acid phosphatase and ribonuclease. Cytochrome oxidase was lowered more than could be accounted for simply by dilution and showed a significant decrease even when related to animal weight. The unsedimentable activities of lysosomal hydrolases were either unchanged or lower than normal.

Results on comatose animals

Deaths, comatose and near-comatose states were recorded only amongst the starved animals and amongst those fed on a deficient diet. In the former group, one animal died after 4 days and another after re-feeding; three were killed on the sixth day of fasting, in a state of deep coma characterized by complete immobility, hypothermia and reduced cardiac and respiratory activity; another animal was already very weak when it was killed. Such was also the case for one of the re-fed animals. Histologically the livers of these comatose animals did not show any greater evidence of damage than the organs of the non-comatose animals in the same group.

The mortality was particularly high in group E. Out of nineteen animals subjected to the deficient diet, seven were discovered dead and five others were found in a comatose state at times between 33 and 87 days after the regimen was started. Both by the suddenness of onset and by the absence of premonitory symptoms, coma and death resembled closely the phenomena described by Glynn & Himsworth (1944). However, in no case were the macroscopic or microscopic signs of massive

hepatic necrosis reported by these authors observed. In several cases, coma or death occurred in animals which had been left without food for a short period of time (less than 24 hr.), and one of the main characteristics of the animals in this group may therefore have been an extreme lack of resistance to fasting. Amongst the five animals which were killed in a comatose state, one was found to have a perforated gastric ulcer, and one other showed none of the biochemical changes observed in all other comatose and precomatose animals. The results obtained on these two animals (E2 and E4) have been presented separately.

In order to find out whether liver damage might decrease the resistance to starvation (a hypothesis suggested by the observations on group E), a number of additional animals were deprived of food, either without pretreatment (group H) or after carbon tetrachloride poisoning (group J). The animals were watched at least every 4 hr. and killed when in a comatose state. For those which were found dead, the time of death was estimated with an error which could not have exceeded 1–2 hr. As shown by Table 2, this experiment revealed that the poisoned animals were actually more resistant to food deprivation than normal ones. This may possibly have been due to their large stores of hepatic fat, which were found to disappear almost entirely during fasting.

The livers of comatose animals in groups H and J were weighed and analysed for cytochrome oxidase, glucose 6-phosphatase, total and unsedimentable acid phosphatase. The results of these determinations, related to animal weight, are shown in Fig. 1, together with the similarly expressed results obtained on the comatose animals of groups B3 (which was combined with group H) and E3, as well as on comparable non-comatose animals. The biochemical picture of the liver of these fourteen comatose animals is strikingly similar: the liver weight was reduced to about two-thirds, cytochrome oxidase to one-third and glucose 6-phosphatase to approximately one-half of their normal values. These changes appear to be largely unaffected by the previous state of the animals. In particular, the animals poisoned with carbon tetrachloride and those on a deficient diet, which presumably already had low glucose 6-phosphatase activities before falling in coma, did not show an additional loss of this enzyme after becoming comatose. Acid phosphatase, on the other hand, was slightly elevated, presumably owing to relative conservation with respect to loss of total animal weight, and was transferred to the extent of about 35 % to the unsedimentable fraction of the homogenates (as compared with 7 % in normal controls).

As shown by Table 1, the behaviour of acid phosphatase is typical of all lysosomal hydrolases, with the exception of β-glucuronidase in group E, which, though similarly released, did not become relatively concentrated during coma. The results observed on the two precomatose animals (B2 and

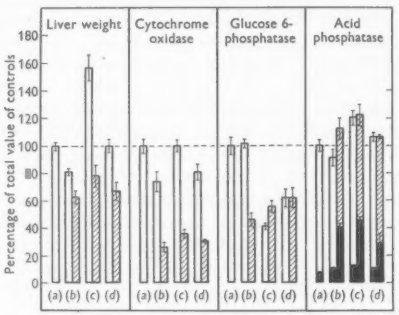


Fig. 1. Biochemical changes associated with coma. Blocks represent means ± S.E.M. All values are related to animal weight and then expressed as percentage of control values. Unfilled blocks, non-comatose animals; cross-hatched blocks, comatose animals; filled-in areas, unsedimentable activity. (*a*) Controls (twenty animals); (*b*) starved animals (eight non-comatose and seven comatose); (*c*) animals poisoned with carbon tetrachloride (eight non-comatose and four comatose as result of subsequent starvation); (*d*) animals on a deficient diet (seven non-comatose and three comatose).

Table 2. *Influence of carbon tetrachloride poisoning on survival during starvation*

Group	H		J	
Treatment	Complete starvation		CCl_4 poisoning as in D, followed by complete starvation	
State of animals	Comatose	Dead	Comatose	Dead
No. of animals	4	4	4	2
Days between beginning of starvation and death or killing in comatose state	6·25 ± 0·15		7·0 ± 0·18	
Difference	0·75 ± 0·23 ($P < 0·01$)			

C2) and on the comatose animal with gastric perforation (E2) also follow the same general pattern. However, animal E4, though killed in what appeared to be a state of deep coma, was biochemically indistinguishable from the non-comatose animals of the same group (E1).

DISCUSSION

The most striking observations concern the comatose and precomatose animals, of which seventeen out of eighteen exhibited the same general enzymic pattern, which was closely reminiscent in its most important aspects of that found by de Duve & Beaufay (1959) after 5–6 hr. of complete ischaemia. As in the latter case, cytochrome oxidase and glucose 6-phosphatase are reduced to respectively one-third and one-half their normal value, whereas all five acid hydrolases are present in unsedimentable form to an extent corresponding to a considerable disruption of the lysosomes. In contrast with the ischaemic livers, the organs of the comatose animals lose weight and nitrogen, presumably by circulatory removal of the products of autolysis, and do not seem to suffer any inactivation of cathepsin and of the nucleases. Consequently, they exhibit a relative concentration of the lysosomal hydrolases, a phenomenon which could not be observed in the ischaemic livers. In this respect, as in their apparent release from the particles with which they are normally associated, the lysosomal enzymes thus show a type of behaviour entirely compatible with their assumed role as agents of the autolytic processes.

In two cases (B2 and C2), the biochemical symptoms preceded those of coma. From this it may be deduced that the severe hepatic lesions revealed by the enzymic assays may take place at a time when the general state of the animal is still relatively unaffected, and could in fact in such cases be responsible for the subsequent onset of coma. Whether this actually did happen in some of the comatose animals cannot be decided, but the case of E4 does at least show that it did not happen in all, since in this animal coma was completely established without appreciable biochemical alterations of the liver.

A slight but significant increase in the amount of lysosomal enzymes released was also observed in all non-comatose animals except in those treated with hydrocortisone. This phenomenon, which corresponds to the situation obtaining after a short period of complete ischaemia, has also been observed in mild post-operative shock (de Duve & Beaufay, 1959) and during treatment with carcinogenic dyes (Deckers-Passau *et al.* 1957). It may therefore be taken as a feature common to all situations where some degree of injury, either associated with localized necrosis or likely to develop into necrosis, is imposed on the tissue. On the other hand, treatment with hydrocortisone, which has been applied successfully in some cases of hepatic injury, appeared to decrease the release of lysosomal enzymes.

As pointed out before (de Duve & Beaufay, 1959), we do not know the exact nature of the intracellular changes responsible for the observed alterations of the lysosomes, but the pathological context with which they are generally associated makes it likely that they correspond to an actual release of hydrolytic activity within the cells. Another problem of interest is that of the mechanism whereby the lysosomes become damaged under the various circumstances studied here. According to Himsworth (1947), obstruction of the intrahepatic circulation may play an important role in the production of necrosis under the influence of toxic or dietetic factors and it is therefore possible that the mechanism of lysosomal damage operative in ischaemic liver may also be involved in some of the present experiments. Other factors could be at work also, for instance a direct injury of the lysosomal membrane by carbon tetrachloride in the experiments with this poison, and by the accumulated bile salts in obstructive jaundice. It is certainly suggestive that glucose 6-phosphatase, which like lysosomes is very sensitive both to carbon tetrachloride and to bile salts (Beaufay & de Duve, 1954), is considerably lowered in both these cases.

An interpretation of the other enzymic changes observed cannot be attempted at the present time. Attention should, however, be drawn to the frequency with which liver tissue exposed to unfavourable conditions responds by an increased content of cathepsin and of acid nucleases. This has been noticed after feeding with carcinogenic dyes or a low-protein diet (Deckers-Passau *et al.* 1957), as well as in several of the present experiments. It should also be noted that the total activities of the various lysosomal enzymes vary in what appears to be a completely independent manner, but that their unsedimentable activities show essentially parallel changes as has also been found to be the case *in vitro*.

It should finally be noted that there was little correlation between the histological and the biochemical findings. Especially where dietary influences were studied (groups B, C and E), few manifest alterations were observed and these were definitely not more pronounced in the livers of comatose animals, despite the dramatic biochemical changes which accompanied the onset of coma. This lack of visible necrosis is perhaps not as surprising as may appear at first sight. As was pointed out above, the enzymic pattern observed

in the comatose animals was similar to that found after 5–6 hr. of complete ischaemia, and this is a state where according to Berenbom, Chang, Betz & Stowell (1955) microscopic alterations are still very slight. There is little reason therefore to question the validity of the biochemical findings, and the conclusion must rather be drawn that enzymic disorganization precedes by probably as much as several hours the appearance of the typical structural features of necrosis. This explanation could also account for our failure to observe, on a limited number of animals, the characteristic signs of dietary necrosis described by Glynn & Himsworth (1944) and by other authors. It is, of course, possible, in view of the observations of Gillman, Gilbert, Gillman & Spence (1952), that the yeast included in the diet may not have been of sufficiently low nutritional value to be necrogenic. However, the high mortality and incidence of coma observed in group E rather suggests that a severe dietary lesion was indeed inflicted but was followed by death too rapidly to express itself morphologically.

Some of the enzymes studied in this work have been previously investigated by other authors under similar conditions, especially after prolonged fasting. The most extensive survey has been made by Allard, de Lamirande & Cantero (1957), who have followed the fate of eleven enzymes during starvation. As in the present work, they have observed a small increase in the specific activity of acid phosphatase and acid ribonuclease, but contrary to the findings of Miller (1948) and to our own they have noted a decrease in the specific activity of cathepsin. Our results on glucose 6-phosphatase confirm those obtained by Weber & Cantero (1954, 1958), Langdon & Weakley (1955) and Ashmore, Hastings, Nesbett & Renold (1956) on fasted animals, and by Weber, Allard, de Lamirande & Cantero (1955) and Ashmore *et al.* (1956) on animals treated with cortical hormones. The only paper relating to the state of the lysosomes is that of Dianzani (1954), who has described what amounts to a complete release of acid phosphatase in hepatic homogenates from animals treated with carbon tetrachloride. In our experiments, this release was much less marked and became essentially complete only in the animals which had been rendered comatose by a subsequent period of prolonged fasting.

SUMMARY

1. A number of properties of the liver have been studied in: (A) normal rats; (B) animals starved for 6 days; (C) animals starved for 6 days and then fed for 8 hr.; (D) animals poisoned with carbon tetrachloride; (E) animals fed on a deficient diet; (F) one animal with ligated bile duct ; (G) animals treated with hydrocortisone; (H) animals rendered comatose by starvation; (J) animals rendered comatose by starvation after carbon tetrachloride poisoning.

2. Microscopic appearance, weight, nitrogen content, cytochrome oxidase and glucose 6-phosphatase activity, total and unsedimentable activities of five lysosomal hydrolases (acid phosphatase, β-glucuronidase, cathepsin, acid ribonuclease and acid deoxyribonuclease) were investigated.

3. The most striking biochemical changes occurred in the animals which were killed in a comatose or precomatose state. Out of eighteen such animals, belonging to groups B, C, E, H and J, seventeen showed the same typical picture characterized by a 30 % greater loss of liver weight and nitrogen than of body weight, an even greater fall of cytochrome oxidase (70 %) and glucose 6-phosphatase (50 %), a conservation with corresponding concentration of the lysosomal hydrolases, accompanied by a considerable transfer of these enzymes from the particulate to the soluble phase of the homogenates.

4. In all non-comatose animals, except those treated with hydrocortisone (group G), there was a significant increase in the proportion of lysosomal hydrolases found in unsedimentable form. Several other significant changes were also found to be associated with the various treatments applied.

5. Few morphological findings of interest were made. In particular, there was no obvious correlation between microscopic appearance and enzymic pattern, even in the livers of comatose animals, which did not show more pronounced alterations than the organs of the non-comatose animals in the same groups.

6. The biochemical results are interpreted as further confirmation of the hypothesis that the opening of lysosomes and consequent release of their internal enzymes plays an important role in the initiation of autolytic and necrotic phenomena, and the lack of correlation between biochemical and morphological findings is taken as an indication that enzymic alterations may precede by as much as several hours the appearance of well-defined microscopic lesions.

This work has been supported by grants from the Centre National de Recherches sur la Croissance normale et pathologique, the Centre National de Recherches Enzymologiques, the Rockefeller Foundation and the Lilly Research Laboratories.

REFERENCES

Allard, C., de Lamirande, G. & Cantero, A. (1957). *Exp. Cell Res.* **13**, 69.

Ashmore, J., Hastings, A. B., Nesbett, F. B. & Renold, A. E. (1956). *J. biol. Chem.* **218**, 77.

Beaufay, H. & de Duve, C. (1954). *Bull. Soc. Chim. biol., Paris*, **36**, 1551.
Berenbom, M., Chang, P. I., Betz, H. E. & Stowell, R. E. (1955). *Cancer Res.* **15**, 1.
Deckers-Passau, L., Maisin, J. & de Duve, C. (1957). *Acta Un. int. Cancr.* **13**, 822.
de Duve, C. & Beaufay, H. (1959). *Biochem. J.* **73**, 610.
de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955). *Biochem. J.* **60**, 604.
Dianzani, M. U. (1954). *Biochim. biophys. Acta*, **14**, 514.
Gillman, J., Gilbert, C., Gillman, T. & Spence, I. (1952). *Amer. J. dig. Dis.* **19**, 201.
Glynn, L. E. & Himsworth, H. P. (1944). *J. Path. Bact.* **66**, 297.
Himsworth, H. P. (1947). In *Lectures on the Liver and its Diseases*, p. 19. Oxford: Blackwell Scientific Publications.
Langdon, R. G. & Weakley, D. R. (1955). *J. biol. Chem.* **214**, 167.
Miller, L. L. (1948). *J. biol. Chem.* **172**, 113.
Weber, G., Allard, C., de Lamirande, G. & Cantero, A. (1955). *Biochim. biophys. Acta*, **16**, 618.
Weber, G. & Cantero, A. (1954). *Science*, **120**, 851.
Weber, G. & Cantero, A. (1958). *Exp. Cell Res.* **14**, 596.

Tissue Fractionation Studies

12. INTRACELLULAR DISTRIBUTION OF SOME DEHYDROGENASES, ALKALINE DEOXYRIBONUCLEASE AND IRON IN RAT-LIVER TISSUE

By H. BEAUFAY,* D. S. BENDALL,† P. BAUDHUIN and C. de DUVE
Department of Physiological Chemistry, University of Louvain, Belgium

(*Received* 24 *March* 1959)

In a previous publication from this Laboratory, a new scheme of fractionation of rat-liver homogenates permitting a more detailed analysis of the large granules has been described (de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955). In the present investigations, this scheme has been applied to a study of the intracellular distribution of a few enzymes which were either known or suspected to be associated with the large granules: the diphosphopyridine nucleotide-linked dehydrogenases of glutamic acid, malic acid and β-hydroxybutyric acid and alkaline deoxyribonuclease (deoxyribonuclease I). In addition, we have studied the distribution of iron to verify the possibility, suggested by electron-microscope observations, that particles rich in ferritin might be concentrated in the same rat-liver fraction as the lysosomes (Novikoff, Beaufay & de Duve, 1956). In all these experiments, cytochrome oxidase, acid phosphatase and glucose 6-phosphatase were measured simultaneously to serve as reference enzymes. Measurements were also made of acid deoxyribonuclease (deoxyribonuclease II), which has been studied before (de Duve *et al.* 1955) but was assayed here by an improved technique. Some of the results obtained have been reported previously in preliminary communications (de Duve & Beaufay, 1957; Baudhuin, 1959).

* Chargé de recherches du FNRS.

† Present address: Department of Biochemistry, Cambridge.

METHODS

The tissue fractionations and determinations of reference enzymes were performed according to the methods described by Appelmans, Wattiaux & de Duve (1955), de Duve *et al.* (1955) and Wattiaux & de Duve (1956), except that cytochrome oxidase was assayed at 25° instead of at room temperature.

Glutamic dehydrogenase was measured by the following modifications of the methods described by Hogeboom & Schneider (1953). For the forward reaction [diphosphopyridine nucleotide (DPN) reduction] the final reaction mixture contained 0·02 M-potassium phosphate buffer, pH 7·7, 0·03 M-nicotinamide, 0·4 mM-sodium cyanide, 0·1% of Triton X-100, mM-cysteine, 13 mM-potassium glutamate, 1·4 mM-DPN and enzyme in a total volume of 3 ml. The reaction was started by the addition of enzyme and the increase in extinction at 340 mμ and 25° was followed in a Beckman model DU spectrophotometer with a reference cell containing all components of the system except glutamate. Triton X-100 was used to release the enzyme; it also causes a reversible inhibition (about 28%) which was ignored in these experiments since the conditions were the same for all fractions. Cysteine could be replaced by mM-ethylenediaminetetra-acetic acid (EDTA).

For the back reaction the reaction mixture contained 0·02 M-potassium phosphate buffer, pH 7·7, 0·03 M-nicotinamide, 0·4 mM-sodium cyanide, 0·1% of Triton X-100, mM-EDTA, 0·01 M-sodium α-oxoglutarate, 0·05 M-ammonium chloride, 0·28 mM-reduced DPN (DPNH) and enzyme in a total volume of 1·8 ml. In order to avoid an initial lag-phase in the reaction, it was found necessary to pre-incubate the enzyme with all components of the reaction mixture except α-oxoglutarate at 25° for 15 min. The reaction was then started by the addition of 0·18 ml. of

0·1 M-sodium α-oxoglutarate and the decrease in extinction at 340 mμ and 25° was followed in the spectrophotometer against a blank containing 0·22 mM-DPNH and all other components of the system except α-oxoglutarate. Under these conditions, the activity was proportional to enzyme concentration.

Malic dehydrogenase was assayed at 25° by following the increase in extinction at 340 mμ in 1·8 ml. of a reaction mixture containing 0·02 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris)–HCl buffer, pH 8·5, mM-EDTA, 0·01 M-sodium cyanide, pH 8·5, 0·03 M-nicotinamide, 0·1% of Triton X-100, 0·7 mM-DPN and 0·05 M-sodium L-malate. This mixture, in which the high concentration of cyanide was necessary to trap the oxalacetate, was allowed to stand at 25° for at least 1 hr. to allow the DPN and cyanide to equilibrate. The reaction was started by the addition of of 0·05–0·1 ml. of the enzyme preparation. The reference cell contained all the components of the system except L-malate. The rates measured in this manner were constant with time up to a change in extinction of 0·1, and proportional to the concentration of enzyme.

The assay of β-hydroxybutyric dehydrogenase was carried out in a similar manner with 0·05 M-sodium DL-β-hydroxybutyrate as substrate. Only the D-isomer will have been oxidized since the L-isomer is only oxidized after conversion into the coenzyme A derivative (Lehninger & Greville, 1953), which could not have been formed under the experimental conditions used. The reaction mixture was made up as for malic dehydrogenase, with the exception that the cyanide concentration was 0·4 mM and that Triton X-100 was omitted. This, as well as a number of other detergents, was found to inhibit the enzyme strongly. Activation of the enzyme was obtained by leaving the preparations in the deep freeze overnight and then, after thawing, treating them for 10 min. in a cooled Blendor (Measuring and Scientific Equipment Ltd., London) turning at full speed. It is not certain that this treatment gave 100% activation, and this may help to explain the low recovery obtained. The progress curve was linear up to a difference in extinction of about 0·25, and the measured rates were proportional to the enzyme concentration.

Alkaline deoxyribonuclease was measured in a manner similar to that adopted for the acid enzyme (de Duve *et al.* 1955; Wattiaux & de Duve, 1956) by measuring spectrophotometrically the amount of acid-soluble oligonucleotides formed at 37° in 2 ml. of a reaction mixture containing 0·05 M-tris–HCl buffer, pH 7·6, mM-magnesium chloride, 0·1% of Triton X-100 and 1·5 mg. of potassium deoxyribonucleate (DNA). When followed in this manner, the reaction progresses along a typical sigmoid curve, shown in Fig. 1. In practice, the assays were performed with three different amounts of enzyme and the value or values falling on the linear part of the reference curve (approximately between 0·5 and 1·5 units) were used to estimate the activity graphically.

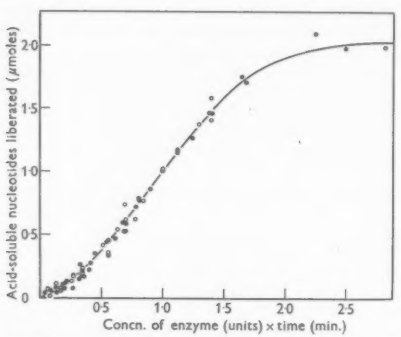


Fig. 1. Kinetics of alkaline deoxyribonuclease. Results of different experiments in which enzyme concentration (●) or incubation time (○) were varied.

Acid deoxyribonuclease was measured as before (Wattiaux & de Duve, 1956), except that the medium contained 0·2 M-KCl. This ensures optimum activity (Rotherham, Schottelius, Irvin & Irvin, 1956; Shack, 1957) and has the additional advantage of inhibiting the alkaline enzyme completely.

Easily detachable iron, representing essentially ferritin and haemosiderin, was determined according to a technique adapted from those of Kitzes, Elvehjem & Schnette (1944) and of Watson (1952). The tissue samples were extracted for 10 min. in a boiling-water bath with 0·5 M-sodium acetate–acetic acid buffer, pH 4·7, 0·06 M-L-ascorbic acid and 0·05% of 2:2′-dipyridyl. The extracts were filtered and their extinction was measured colorimetrically with a light-filter of maximal transmittance at 500 mμ. Total iron was determined similarly after digestion in conc. H_2SO_4.

In conformity with previous publications (de Duve *et al.* 1955) units of enzymic activity are defined as the amount of enzyme causing the decomposition of 1 μmole of substrate/min. under the conditions of the assays (except for cytochrome oxidase, see Appelmans *et al.* 1955). In these calculations, a molar-extinction coefficient at 340 mμ of $6{\cdot}22 \times 10^6$ cm.^{2} mole^{-1} was used for DPNH (Horecker & Kornberg, 1948) and the breakdown of DNA was evaluated in mononucleotides on the basis of an average molar-extinction coefficient at 260 mμ of $8{\cdot}5 \times 10^6$ cm.^{2} mole^{-1} (Stimson & Reuter, 1945).

The materials used in these experiments were the following: Triton X-100 (Rohm and Haas, Co., Philadelphia, Pa., U.S.A.); DL-sodium β-hydroxybutyrate (British Drug Houses Ltd.), DPN (Sigma Chemical Co., St Louis, Mo., U.S.A.); tris (Sigma Chemical Co.); L-glutamic acid (Pfanstiehl Chemical Co., Waukegan, Ill., U.S.A.); L-cysteine hydrochloride (Hofmann–La Roche and Co., Basle, Switzerland); α-oxoglutaric acid (Sigma Chemical Co.). Other substrates were those described by de Duve *et al.* (1955).

RESULTS

Distribution of enzymes

The enzyme distributions found are listed in Table 1 and shown graphically in Fig. 2. The results obtained on the reference enzymes and on acid deoxyribonuclease were essentially similar to those recorded previously (de Duve *et al.* 1955), except that the nuclear fraction was more heavily contaminated with cytoplasmic components. There is also a somewhat greater variability in the values obtained for the heavy mitochondrial (M) and light

Table 1. *Intracellular distribution of enzymes*

Absolute values are given in mg./g. for nitrogen; in units/g. for enzymes. Statistics refer to the means±S.D. These are given rather than the S.E.M. since they are a measure of individual variability, the most interesting information in the present case. E, Cytoplasmic extract; N, nuclear fraction; M, heavy mitochondrial fraction; L, light mitochondrial fraction; P, microsomal fraction; S, final supernatant.

	No. of experiments	Absolute values E+N	Percentage values					
			E+N	N	M	L	P	S	Recovery
Nitrogen	4	35·4±5·2	100	22·3±4·3	16·3±2·3	5·4±1·8	22·3±3·8	34·1±3·8	100·4±4·0
Cytochrome oxidase	3	21·2±7·4	100	14·7±4·5	50·2±4·8	18·4±8·1	6·1±3·1	—	89·4±9·2
Glutamic dehydrogenase	2	13·4 (59·4)*	100	17·9±8·1	56·3±8·6	14·4±10·1	4·9±0·3	2·2±1·4	95·7±5
Malic dehydrogenase	1	53·4	100	16·4	60·0	11·4	1·5	11·2	100·5
β-Hydroxybutyric dehydrogenase	1	11·4	100	14·4	54·7	6·2	4·4	0·3	80·0
Alkaline deoxyribonuclease	3	0·927±0·26	100	20·6±3·6	52·8±8·0	12·7±7·3	15·7±5·8	3·6±6·2	105·4±9·1
Acid phosphatase	4	6·40±0·98	100	11·9±3·1	17·2±6·9	33·2±5·5	23·2±4·6	9·7±3·4	95·2±4·2
Acid deoxyribonuclease	3	0·716±0·20	100	17·9±7·5	25·4±3·8	32·0±15·4	15·1±1·6	6·9±6·9	97·3±13·4
Glucose 6-phosphatase	4	22·2±4·7	100	17·1±6·5	3·2±0·6	3·7±0·9	80·6±17·6	2·0±0·8	106·6±17·6

* Value in parentheses is for DPNH oxidation (back reaction); the other value is for DPN reduction (forward reaction).

mitochondrial (L) fractions in individual experiments. This is due to slight differences in the manner in which the supernatant was decanted from the M sediment.

As could already be suspected from the data of Hogeboom & Schneider (1953) and of Allard, de Lamirande & Cantero (1957), the distribution of glutamic dehydrogenase paralleled that of cytochrome oxidase. So did those of malic dehydrogenase, β-hydroxybutyric dehydrogenase and alkaline deoxyribonuclease, except for a 10% excess of alkaline deoxyribonuclease in the microsomes and of malic dehydrogenase in the soluble fraction.

Distribution of iron

In Table 2 and Fig. 3 are shown the results of five experiments in which the distribution of easily detachable iron was measured. In conformity with the data of Thiers & Vallee (1957) and of Kuff & Dalton (1957), the largest proportion of the hepatic iron was recovered in the final supernatant, from which fraction Kuff & Dalton (1957) have been able to separate ferritin particles by prolonged ultracentrifuging. The remainder of the iron was distributed diffusely amongst the particulate fractions, with, however, a small but significant peak in the light mitochondrial fraction (L), which is richest in lysosomes (Fig. 3). As shown by Fig. 4, there is a distinct correlation between the iron concentration and the specific activity of acid phosphatase in this fraction, an indication that the two components are purified simultaneously. In

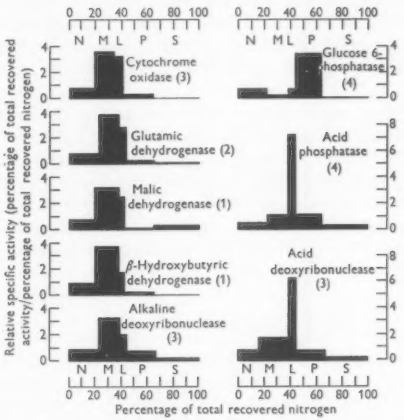

0 20 40 60 80 100
N M L P S
Cytochrome oxidase (3)
Glucose 6-phosphatase (4)
Glutamic dehydrogenase (2)
Acid phosphatase (4)
Malic dehydrogenase (1)
β-Hydroxybutyric dehydrogenase (1)
Acid deoxyribonuclease (3)
Alkaline deoxyribonuclease (3)
Relative specific activity (percentage of total recovered activity/percentage of total recovered nitrogen)
Percentage of total recovered nitrogen

Fig. 2. Distribution patterns of enzymes. Ordinate: mean relative specific activity of fractions. Abscissa: fractions are represented by their relative nitrogen content, in the order in which they are isolated. Numbers in parentheses refer to numbers of experiments. Abbreviations are defined in Table 1.

Table 2. *Intracellular distribution of iron*

Results of five experiments: absolute values are given in mg./g. for nitrogen and iron; in units/g. for enzymes. Statistics refer to the means ± S.D. Abbreviations are defined in Table 1.

Component	Absolute values E + N	Percentage values E + N	N	M	L	P	S	Recovery
Nitrogen	34·9 ± 2·7	100	15·8 ± 3·6	17·8 ± 2·1	3·7 ± 0·5	20·8 ± 2·6	35·7 ± 2·1	93·8 ± 2·9
Cytochrome oxidase	26·1 ± 6·0	100	13·7 ± 7·3	57·4 ± 5·6	13·3 ± 9·0	7·2 ± 1·9	—	91·6 ± 11·8
Acid phosphatase	6·54 ± 0·66	100	6·5 ± 1·3	22·5 ± 4·2	37·8 ± 2·6	18·1 ± 3·2	14·5 ± 2·0	99·4 ± 2·5
Glucose 6-phosphatase	22·0 ± 2·1	100	7·6 ± 1·7	2·5 ± 0·6	2·0 ± 0·4	85·2 ± 12·7	2·9 ± 1·4	100·2 ± 12·7
Easily detachable iron	0·281 ± 0·88	100	5·3 ± 0·9	6·6 ± 1·8	4·4 ± 0·8	10·9 ± 2·5	68·2 ± 4·3	95·4 ± 3·5
Total iron*	0·244 (0·211)	100	9·4 (5·1)	5·6 (6·3)	5·2 (4·9)	12·3 (9·0)	65·4 (75·1)	97·9 (100·4)

* Result of a single experiment. The values for easily detachable iron in the same experiment are given in parentheses.

one of the above experiments, the distribution of total iron was also measured. As indicated in Table 2, excess of iron, originating mostly from haemoglobin, was found essentially in the nuclear and microsomal fractions.

In another experiment, the animal was given 20 mg. of iron in the form of saccharated iron

Fig. 3. Distribution patterns of easily detachable iron and of acid phosphatase. Ordinate: mean relative specific content or activity of fractions. Abscissa: fractions are represented by their relative nitrogen content, in the order in which they are isolated. Results of five experiments. Abbreviations are defined in Table 1.

Fig. 4. Correlation between relative specific iron content and relative specific acid phosphatase activity of light mitochondrial (L) fractions. Results from five fractionations and three other purification experiments.

Table 3. *Intracellular distribution of easily detachable iron after administration of iron*

Fractionation of liver from rat injected with 20 mg. of iron in the form of saccharated iron oxide 3 days before being killed. Absolute values are given in mg./g. for nitrogen and iron, in units/g. for enzymes. Abbreviations are defined in Table 1.

Component	Absolute values E+N	Percentage values E+N	N	M	L	P	S	Recovery
Nitrogen	27·7	100	15·2	11·8	3·8	18·3	42·2	91·3
Cytochrome oxidase	17·8	100	16·6	43·6	11·7	10·6	—	82·5
Acid phosphatase	3·49	100	7·6	19·7	28·2	20·9	13·7	90·1
Glucose 6-phosphatase	8·05	100	5·6	1·8	1·3	66·3	0·5	75·5
Iron	1·74	100	31·7	19·8	3·3	6·4	32·9	94·1

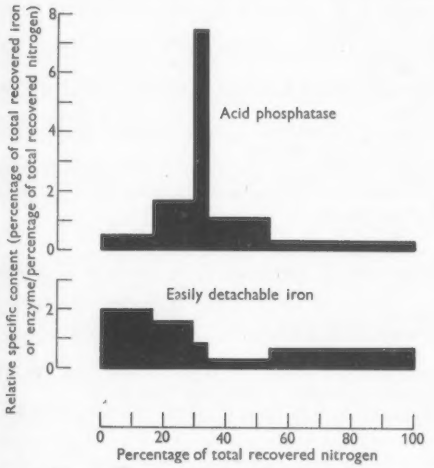


Fig. 5. Distribution patterns of easily detachable iron and of acid phosphatase in liver of rat injected with 20 mg. of iron in the form of saccharated iron oxide 3 days before being killed. For explanation see Fig. 3.

oxide (Hausmann Laboratories, Saint-Gall, Switzerland) administered intravenously 3 days before it was killed. The results obtained on the liver of this animal are given in Table 3 and Fig. 5. They show that the extra iron tends to accumulate in the form of gross haemosiderin particles, which, from the data of Gabrio, Shoden & Finch (1953), may be expected to sediment largely with the nuclei.

DISCUSSION

The method of fractionation applied in this work was primarily devised to distinguish between truly mitochondrial enzymes and others, such as the lysosomal hydrolases and uricase, which are also concentrated in the mitochondrial fraction when the classical method is followed (de Duve *et al.* 1955). The truly mitochondrial enzymes, like cytochrome oxidase, occur in larger amounts and with a higher specific activity in the heavy (M) than in the light (L) mitochondrial fraction, whereas the reverse is true for the others, which follow the distribution of acid phosphatase.

The results obtained thus indicate that the three dehydrogenases studied, as well as alkaline deoxyribonuclease, are essentially located in the mitochondria, whereas the acid deoxyribonuclease is associated with the lysosomes. As far as the dehydrogenases are concerned the observed association is in conformity with the well-known metabolic properties of mitochondria. More surprising are the results obtained on alkaline deoxyribonuclease. It now appears that of the two intracellular enzymes known to attack DNA, one is present in the lysosomes and the other in the mitochondria.

The presence of excess of iron in fraction L and the correlation observed between the iron content of this fraction and its acid phosphatase activity must be considered in conjunction with the observation that lysosome-rich fractions from rat liver contain a large number of particles (peribiliary dense bodies) loaded with electron-dense granules (Novikoff *et al.* 1956). The present findings confirm the interpretation based on morphological criteria that these electron-dense granules are made of ferritin. They afford additional support to the possibility that lysosomes and peribiliary dense bodies may be identical. Until purer fractions can be isolated this identity must, however, remain hypothetical.

SUMMARY

1. The intracellular distribution of a number of components has been investigated in rat liver, according to a fractionation scheme in which the mitochondrial fraction is divided into two subfractions. In most experiments, cytochrome oxidase, acid phosphatase and glucose 6-phosphatase were measured as reference enzymes for mitochondria, lysosomes and microsomes respectively.

2. Glutamic, malic and β-hydroxybutyric dehydrogenases, together with alkaline deoxyribonuclease (DNase I), were found to be associated essentially with the true mitochondria. In addition, the microsomal fraction contained about 10% excess of alkaline deoxyribonuclease over what could be expected from mitochondrial contamination, and a similar excess of malic dehydrogenase was found in the final supernatant. As observed before, acid deoxyribonuclease (DNase II), which was measured here by an improved technique, showed a lysosomal distribution.

3. About two-thirds of the easily detachable iron of liver tissue was recovered in the final supernatant. The remainder was distributed diffusely amongst all particulate fractions, with a significant peak in the light mitochondrial fraction, which is richest in lysosomes. The iron content of this fraction was correlated with its acid phosphatase activity. These results support the contention, based on electron-microscope observations, that particles loaded with ferritin are concentrated together with lysosomes.

4. After administration of iron, the iron content of all fractions was augmented; the greatest increase occurred in the nuclear and heavy mitochondrial fractions, in which the iron was probably present in the form of gross haemosiderin granules.

This work was supported by grants from the Centre National de Recherches sur la Croissance normale et pathologique, the Centre National de Recherches Enzymologiques, the Rockefeller Foundation and the Lilly Research Laboratories. One of us (D.S.B.) is indebted to the Belgian Government for a scholarship and to the Administrators of the H.E. Durham Fund, King's College, Cambridge, for a grant.

REFERENCES

Allard, C., de Lamirande, G. & Cantero, A. (1957). *Exp. Cell Res.* **13**, 69.
Appelmans, F., Wattiaux, R. & de Duve, C. (1955). *Biochem. J.* **59**, 438.
Baudhuin, P. (1959). *Arch. int. Physiol. Biochim.* **67**, 106.
de Duve, C. & Beaufay, H. (1957). *Arch. int. Physiol. Biochim.* **65**, 160.
de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955). *Biochem. J.* **60**, 604.
Gabrio, B. W., Shoden, A. & Finch, C. A. (1953). *J. biol. Chem.* **204**, 815.
Hogeboom, G. H. & Schneider, W. C. (1953). *J. biol. Chem.* **204**, 233.
Horecker, B. L. & Kornberg, A. (1948). *J. biol. Chem.* **175**, 385.
Kitzes, G., Elvehjem, C. A. & Schnette, H. A. (1944). *J. biol. Chem.* **155**, 653.
Kuff, E. L. & Dalton, A. J. (1957). *J. Ultrastruct. Res.* **1**, 62.
Lehninger, A. L. & Greville, G. D. (1953). *Biochim. biophys. Acta*, **12**, 188.
Novikoff, A. B., Beaufay, H. & de Duve, C. (1956). *J. biophys. biochem. Cytol.* **2**, Suppl. 179.
Rotherham, J., Schottelius, D. D., Irvin, J. L. & Irvin, E. M. (1956). *J. biol. Chem.* **223**, 817.
Shack, J. (1957). *J. biol. Chem.* **226**, 573.
Stimson, M. M. & Reuter, M. A. (1945). *J. Amer. chem. Soc.* **67**, 487.
Thiers, R. E. & Vallee, B. L. (1957). *J. biol. Chem.* **226**, 911.
Watson, D. (1952). *J. med. Lab. Tech.* **10**, 3.
Wattiaux, R. & de Duve, C. (1956). *Biochem. J.* **63**, 606.

Tissue Fractionation Studies

13. ANALYSIS OF MITOCHONDRIAL FRACTIONS FROM RAT LIVER BY DENSITY-GRADIENT CENTRIFUGING

By H. BEAUFAY,* D. S. BENDALL,† P. BAUDHUIN, R. WATTIAUX and C. DE DUVE
Department of Physiological Chemistry, University of Louvain, Belgium

(*Received* 24 *March* 1959)

In the investigations described in this paper an attempt has been made to analyse in a more detailed manner the distribution of various enzymes in mitochondrial fractions from rat liver, with the help of density-gradient centrifuging. The results obtained, which have been briefly reported before (Beaufay, Baudhuin & Bendall, 1958; Baudhuin, 1959), fully confirm the distinction between mitochondria and lysosomes and provide additional information about both groups of particles.

Density-gradient centrifuging, especially when applied to cell particles, requires specialized equipment and fairly elaborate mathematical treatments. These aspects, as well as the theoretical bases of the method, have recently been examined in detail by de Duve, Berthet & Beaufay (1959). To avoid lengthy duplication of material, we must refer to the above mentioned paper for all details which are not strictly essential to the understanding of the present account.

* Chargé de recherches du FNRS.
† Present address: Department of Biochemistry, Cambridge.

METHODS

Density-gradient centrifuging

All experiments were performed on washed mitochondrial fractions prepared according to Appelmans & de Duve (1955). When the gradients were made with deuterium oxide, the last washing was carried out with 0·25 M-sucrose in heavy water and followed by centrifuging at 800 000 *g*. min. Equal quantities of the particles were added to two solutions of different density and these two suspensions were then used to make a linear gradient with the help of a twin-syringe machine. Thus at the beginning of the experiment the particles themselves were always distributed homogeneously within the tube whereas other components were not. In most experiments the equivalent of 0·6 g. of original tissue was suspended in this manner in a total volume of 5 ml. The tubes had a cross-sectional area of 1·22 cm.² and the column of fluid could thus be assimilated (neglecting the sphericity of the bottom) to a cylinder 4·1 cm. high. In calculating the physical properties of the suspension media, it was assumed that particles from 1 g. of liver occupy a volume of 0·25 ml. in 0·25 M-sucrose and that they lose 0·075 and 0·125 ml. of water respectively in 0·5 M-sucrose and in highly hyperosmotic sucrose, as a result of osmotic dehydration (Tedeschi & Harris, 1955).

The tubes were centrifuged with the swinging-bucket SW-39 rotor in a Spinco model L preparative ultracentrifuge equipped with special devices designed to ensure slow acceleration and deceleration, to prevent wobbling of the rotor at low speed and to record the angular velocity.

After centrifuging, the column of fluid was divided into approximately ten successive portions, of height varying between 0·2 and 1·5 cm. depending on the observed distribution of material. A special cutter was used for this purpose and the amount of fluid separated by the knife was sucked off each time, weighed and then diluted with a known volume of a solution containing mM-sodium bicarbonate, mM-ethylenediaminetetra-acetic acid and 0·01% of Triton X-100. The volume of the undiluted fractions was calculated by dividing their weight by the average density corresponding to their position in the gradient. Thus the precise dilution could be known for each fraction. In addition, their theoretical volume was estimated from the height of the cut and the cross-sectional area of the tube. As a rule, the difference between theoretical and measured volume, representing losses due to leakage and surface contamination of the cutter and pipette, amounted to about 10%. The sediment adhering to the bottom of the tube was also recovered and resuspended in a known volume of the diluent described above.

All these operations were performed with the equipment described by de Duve *et al.* (1959) and at a temperature only slightly above the freezing point of the solvent (0° for water and 3·82° for deuterium oxide). Some degree of heating of the rotor during prolonged centrifuging at high speed could, however, not be prevented. The conditions of centrifuging are expressed by the time integral of the squared angular velocity: $W = \int_0^t \omega^2 \, dt$.

Analytical procedures

Cytochrome oxidase was measured as described by Appelmans, Wattiaux & de Duve (1955). Glutamic and malic dehydrogenase as well as alkaline deoxyribonuclease were assayed according to Beaufay, Bendall, Baudhuin & de Duve (1959). Except in one case, the back reaction (oxidation of reduced diphosphopyridine nucleotide) was used in all glutamic dehydrogenase assays. Uricase was determined as reported by de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955), but at 37° and in the presence of 0·1% of Triton X-100 to increase the sensitivity of the assay. For the lysosomal hydrolases, the methods of Wattiaux & de Duve (1956) were adapted to the use of long incubation periods (24 hr.) and small amounts of enzyme. All substrate mixtures contained 0·01% of sodium thiomersalate (Eli Lilly and Co., Indianapolis, Ind., U.S.A.) to prevent bacterial contamination. With acid phosphatase and β-glucuronidase, the preliminary fixation and filtration were omitted and the analytical procedures were carried out immediately. For cathepsin and the nucleases, the usual procedure was followed. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Calculation and presentation of results

As a rule, the results are given in the form of a graph and a complementary table. In the graphs, the data are expressed in terms of the ratio of the concentration found (C) to the initial concentration (C_i) and plotted graphically as a function either of the mean distance separating the analysed fraction from the meniscus or of the corresponding density. The concentration C was obtained by multiplying the result of the analysis performed on the diluted sample by the dilution factor determined as explained above. The initial concentration C_i was calculated by dividing the total amount recovered by the volume of suspension. To compute the total amount recovered, each value of C was multiplied by the corresponding theoretical volume and these products were added together and with the total amount found in the sediment. By calculating C_i in this manner, the ratios C/C_i were automatically corrected for faulty recoveries.

The amounts found in the sediments and in some of the top fractions are not represented in the graphs, since they correspond to discontinuities in the gradient. They are given separately in the tables, together with the actual recoveries. These values are expressed as percentages of the total amounts determined on a mixture of equal parts of the two suspensions used to prepare the gradients.

RESULTS

Sedimentation velocity

In these experiments, the density of the suspension medium was kept below that of the particles at all levels and the centrifuging conditions were chosen so as to cause incomplete sedimentation of the particles, the gradient serving simply a stabilizing purpose. The gradient chosen extended linearly from 0·25 to 0·5 M-sucrose over a radial distance of 5·5–9·6 cm. In this system, as shown by de Duve *et al.* (1959), the velocity of sedimentation of osmotically active particles having the same equilibrium density (1·22, see below) as rat-liver

mitochondria is practically independent of their position and given in first approximation by

$$\frac{dx}{dt} = 5{\cdot}75 . S_{s} . \omega^{2}, \quad (1)$$

in which x is the radial distance from the axis, S_s is the standard sedimentation coefficient (in 0·25 M-sucrose at 0°) and ω is the angular velocity.

By integration and with the meniscus at 5·5 cm., (1) gives:

$$x - 5{\cdot}5 = 5{\cdot}75 . S_{s} \int_{0}^{t} \omega^{2}\, dt = 5{\cdot}75 . S_{s} . W. \quad (2)$$

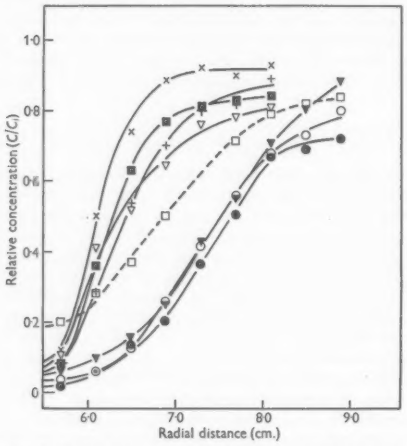


Fig. 1. Sedimentation boundaries of particle-bound enzymes. Linear gradient of 0·25–0·5 M-sucrose over radial distance of 5·5–9·6 cm. $W = 2{\cdot}63 \times 10^{8}$ rad.^{2} sec.$^{-1}$. Complementary data are given in Table 1. ●, Cytochrome oxidase; ○, glutamic dehydrogenase; ▼, alkaline deoxyribonuclease; ×, acid phosphatase; +, β-glucuronidase; ■, cathepsin; ▽, acid deoxyribonuclease; □, protein.

Table 1. *Complementary data on experiment of Fig.* 1

Values given refer to the activity of the sediment and to the sum of the activities of all the fractions, expressed in percentages of the total activity included in the gradient.

Enzyme	Percentage of total activity in gradient: Sediment	Percentage of total activity in gradient: Total recovery
Cytochrome oxidase	71·6	108·0
Glutamic dehydrogenase	65·2	100·5
Alkaline deoxyribonuclease	54·9	88·2
Acid phosphatase	34·6	104·0
β-Glucuronidase	40·5	96·1
Cathepsin	40·1	98·6
Acid deoxyribonuclease	45·6	99·3
Protein	56·7	107·2

The results of three such experiments performed under comparable conditions, and of a fourth carried out with about twice the total force, are shown in Figs. 1–4 and in Tables 1–4. In Fig. 5 are represented the sedimentation-coefficient distribution curves, obtained by differentiating the observed sedimentation boundaries according to de Duve *et al.* (1959). An approximate size scale, calculated on the assumption that the particles are spherical and have a density of 1·10 in 0·25 M-sucrose, is appended for comparison with the results of other workers. For reasons which are

Table 2. *Complementary data on experiment of Fig.* 2

Values given refer to the activity of the sediment and to the sum of the activities of all the fractions, expressed in percentages of the total activity included in the gradient.

Enzyme	Percentage of total activity in gradient: Sediment	Percentage of total activity in gradient: Total recovery
Cytochrome oxidase	51·3	98·2
Glutamic dehydrogenase	50·0	96·8
Acid phosphatase	25·4	96·3
β-Glucuronidase	31·1	93·3
Cathepsin	25·2	89·7
Acid deoxyribonuclease	24·5	79·6
Uricase	26·4	96·5
Protein	43·7	92·1

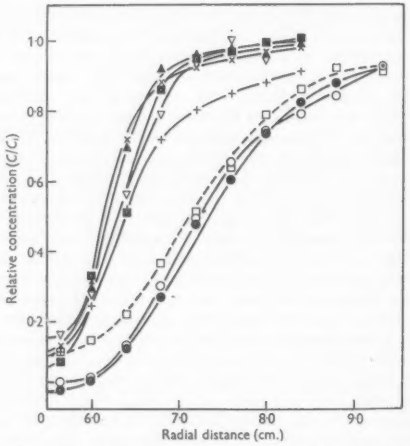


Fig. 2. Sedimentation boundaries of particle-bound enzymes. Experimental conditions were as in Fig. 1. $W = 2{\cdot}69 \times 10^{8}$ rad.^{2} sec.$^{-1}$. Complementary data are given in Table 2. ●, Cytochrome oxidase; ○, glutamic dehydrogenase; ×, acid phosphatase; +, β-glucuronidase; ■, cathepsin; ▽, acid deoxyribonuclease; ▲, uricase; □, protein.

explained in the discussion, the losses evidenced by the decrease below unity of the plateau values of C/C_i have been attributed to agglutination or adsorption artifacts rather than to rapid sedimentation after side-wall impaction. Consequently, in the computation of the distribution curves, the geometrical factor γ (de Duve *et al.* 1959) has been taken equal to zero and the ordinate values have been corrected for losses by multiplying by the inverse of the plateau value of C/C_i. Thus in view of equation (2):

$$\text{Abscissa: } S_s = \frac{(x-5{\cdot}5)}{5{\cdot}75W}. \qquad (3)$$

$$\text{Ordinate: } F(S_s) = \frac{dC}{C_i dS_s} = \frac{dC}{C_i dx}.5{\cdot}75.\frac{C_i}{C\ (\text{plateau})}. \qquad (4)$$

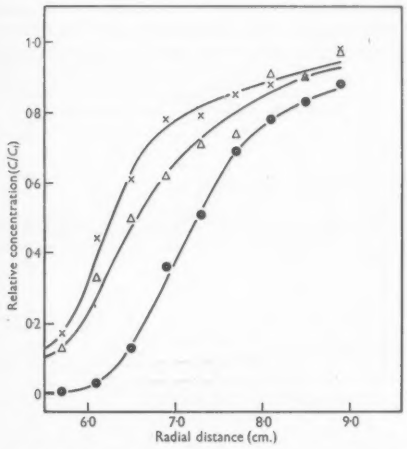


Fig. 3. Sedimentation boundaries of particle-bound enzymes. Experimental conditions were as in Fig. 1. $W = 2{\cdot}70 \times 10^8$ rad.^{2} sec.$^{-1}$. Complementary data are given in Table 3. ●, Cytochrome oxidase; ×, acid phosphatase; △, acid ribonuclease.

Table 3. *Complementary data on experiment of Fig.* 3

Values given refer to the activity of the sediment and to the sum of the activities of all the fractions expressed in percentages of the total activity included in the gradient.

Enzyme	Percentage of total activity in gradient: Sediment	Percentage of total activity in gradient: Total recovery
Cytochrome oxidase	61·5	101·5
Acid phosphatase	34·3	101·4
Acid ribonuclease	44·1	84·2

It is clear from the results obtained in these experiments that the enzymes studied may be divided into two groups: a heavier, mitochondrial group comprising cytochrome oxidase, glutamic dehydrogenase and alkaline deoxyribonuclease, and a more slowly sedimenting one including uricase and the five lysosomal acid hydrolases. The shape of the distribution curves varies little from one enzyme to the other in the former group; it is more variable in the latter, being sharpest for uricase and most spread out for acid ribonuclease and β-glucuronidase. The comparisons of Fig. 5

Table 4. *Complementary data on experiment of Fig.* 4

Values given refer to the activity of the sediment and to the sum of the activities of all the fractions, expressed in percentages of the total activity included in the gradient.

Enzyme	Percentage of total activity in gradient: Sediment	Percentage of total activity in gradient: Total recovery
Cytochrome oxidase	85·0	96·1
Glutamic dehydrogenase	85·1	102·2
Acid phosphatase	42·4	99·4
β-Glucuronidase	51·1	91·5
Cathepsin	46·5	91·9
Acid deoxyribonuclease	48·4	99·7
Uricase	33·6	80·0

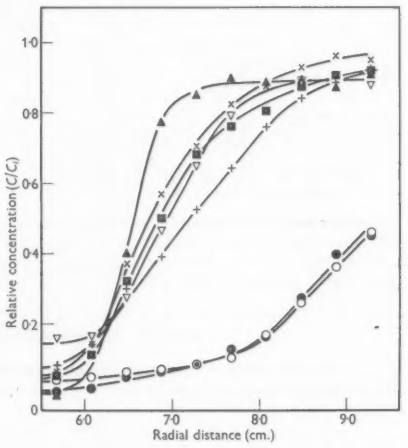


Fig. 4. Sedimentation boundaries of particle-bound enzymes. Experimental conditions were as in Fig. 1. $W = 5{\cdot}03 \times 10^8$ rad.^{2} sec.$^{-1}$. Complementary data are given in Table 4. ●, Cytochrome oxidase; ○, glutamic dehydrogenase; ×, acid phosphatase; +, β-glucuronidase; ■, cathepsin; ▽, acid deoxyribonuclease; ▲, uricase.

also make it possible to judge the reproducibility of the method which, though not perfect, may, however, be considered as fairly satisfactory.

Density equilibration

In these experiments, the limits of the gradient were chosen so as to encompass the equilibrium density of the particles. Centrifuging was pursued until, as predicted by de Duve *et al.* (1959), particles of the size of mitochondria had moved up or down the tube to a position corresponding closely to their own density. Particles of smaller size were not expected to be equilibrated as completely, but the duration of centrifuging necessary to achieve this purpose would have been prohibitive.

In Fig. 6 and Table 5 are given the results of such an experiment perform.ed in aqueous sucrose, and the results of two others, in which deuterium oxide was used as solvent, are shown in Figs. 7 and 8 and in Tables 6 and 7. In all three experiments, the mitochondrial group of enzymes stands out clearly from the others and shows almost perfect parallelism with the total proteins. The five lysosomal hydrolases show similar flattened curves which are progressively shifted towards higher densities in the order: acid phosphatase, β-glucuronidase, cathepsin, acid ribonuclease and acid deoxyribonuclease. These differences are not very marked in water but are exaggerated considerably in deuterium oxide, which causes a much greater shift of the equilibrium density of lysosomes than of mitochondria. This shift is particularly marked for uricase, which also shows a sharper curve than the lysosomal hydrolases.

As shown by the results of Table 8, it is possible by an appropriate choice of conditions to separate in heavy water a sediment practically free of mitochondria and in which the lysosomal enzymes and

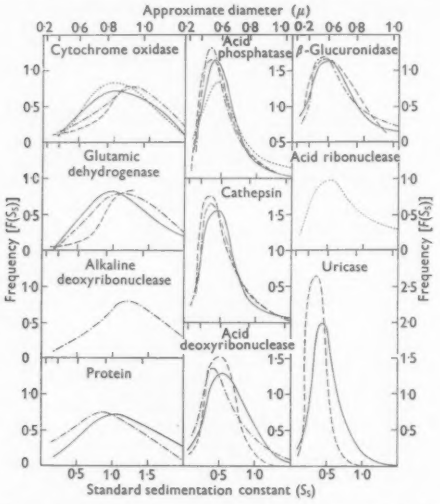


Fig. 5. Sedimentation-coefficient distribution curves derived from Figs. 1–4 as described in the text. —·—, Curves of Fig. 1; — —, curves of Fig. 2; ----, curves of Fig. 3; — —, curves of Fig. 4.

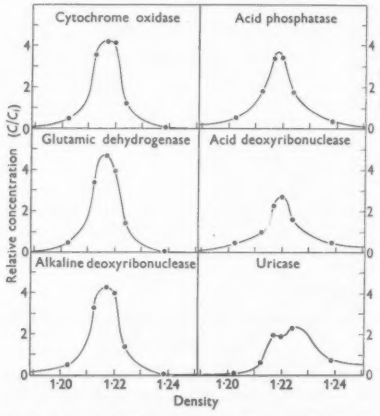


Fig. 6. Density equilibration in a linear gradient of 1·42–1·88 M-sucrose in water over a radial distance of 5·5–9·6 cm. $W = 1{\cdot}2 \times 10^{11}$ rad.^{2} sec.$^{-1}$ (2 hr. at 39 000 rev./min.). Complementary data are given in Table 5.

Table 5. *Complementary data on experiment of Fig.* 6

Values given refer to the activities of the top fraction and of the sediment, and to the sum of the activities of all the fractions, expressed in percentages of the total activity included in the gradient.

	Percentage of total activity in gradient		
Enzyme	Top fraction	Sediment	Total recovery
Cytochrome oxidase	3·4	0·1	104·5
Glutamic dehydrogenase	2·4	0·2	—
Alkaline deoxyribonuclease	5·0	1·3	123·6
Acid phosphatase	7·8	3·6	94·0
Acid deoxyribonuclease	6·1	13·9	87·3
Uricase	1·5	24·4	74·5

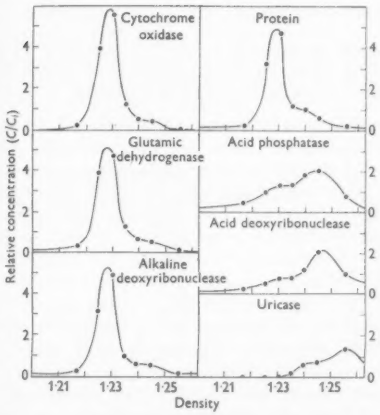


Fig. 7. Density equilibration in a linear gradient of 0·87–1·46 M-sucrose in D_2O over a radial distance of 5·5–9·6 cm. $W = 1{\cdot}8 \times 10^{11}$ rad.^{2} sec.$^{-1}$ (3 hr. at 39 000 rev./min.). Complementary data are given in Table 6.

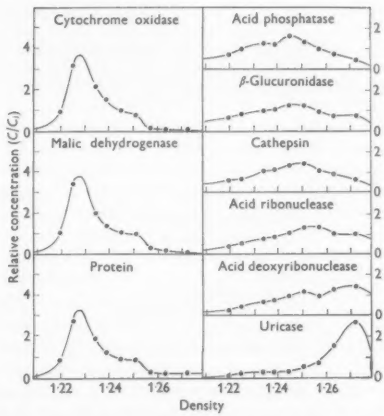


Fig. 8. Density equilibration in a linear gradient of 0·95–1·61 M-sucrose in D_2O over a radial distance of 5·5–9·6 cm. $W = 1{\cdot}8 \times 10^{11}$ rad.^{2} sec.$^{-1}$ (3 hr. at 39 000 rev./min.). Complementary data are given in Table 7.

Table 6. *Complementary data on experiment of Fig.* 7

Values given refer to the activities of the top fraction and of the sediment, and to the sum of the activities of all the fractions, expressed in percentages of the total activity included in the gradient.

	Percentage of total activity in gradient		
Enzyme	Top fraction	Sediment	Total recovery
Cytochrome oxidase	1·4	0·1	122·0
Glutamic dehydrogenase	1·1	0·2	109·0
Alkaline deoxyribonuclease	6·0	6·1	142·5
Acid phosphatase	8·3	4·8	98·2
Acid deoxyribonuclease	7·2	29·2	123·0
Uricase	0	33·5	59·5
Protein	3·0	2·5	130·7

Table 7. *Complementary data on experiment of Fig.* 8

Values given refer to the activities of the top fraction and of the sediment, and to the sum of the activities of all the fractions, expressed in percentages of the total activity included in the gradient.

	Percentage of total activity in gradient		
Enzyme	Top fraction	Sediment	Total recovery
Cytochrome oxidase	4·2	0	—
Malic dehydrogenase	4·0	0·1	92·2
Acid phosphatase	12·2	3·1	102·7
β-Glucuronidase	13·1	3·5	100·3
Cathepsin	11·1	5·1	99·7
Acid ribonuclease	7·6	11·2	102·8
Acid deoxyribonuclease	7·9	14·0	93·5
Uricase	1·7	14·6	95·7
Protein	4·1	1·6	98·0

Table 8. *Isolation of a lysosome-rich sediment*

Sediment from mitochondrial fraction centrifuged for 3 hr. at 39 000 rev./min. ($W = 1{\cdot}8 \times 10^{11}$ rad.^{2} sec.$^{-1}$) in linear gradient of 0·9–1·51 M-sucrose in D_2O over radial distance of 5·5–9·6 cm. Values given refer to the activity of the sediment and to the sum of the activities of all the fractions, expressed in percentages of the total activity included in the gradient.

Enzyme	Percentage of total activity in gradient: Sediment	Percentage of total activity in gradient: Total recovery	Percentage of recovered activity / Percentage of recovered protein
Cytochrome oxidase	0·1	92·5	0·24
Acid phosphatase	9·7	98·2	22·4
β-Glucuronidase	13·0	92·5	31·8
Cathepsin	10·9	91·9	26·7
Acid ribonuclease	10·8	95·8	25·5
Acid deoxyribonuclease	11·9	80·9	33·3
Uricase	35·4	102·4	78·2
Protein	0·4	90·4	1·0

particularly uricase are purified to a considerable extent. Remembering that the original preparation used in this experiment contained about 70% of the total tissue content of these enzymes purified approximately threefold, we find that the hydrolases of the sediment were purified 75- to 100-fold with a 10% yield, and uricase some 250-fold with a 25% yield.

DISCUSSION

Technical and theoretical considerations

The experiments reported in this paper are the first to be performed according to the principles set forth in the theoretical paper of de Duve *et al.* (1959) and it is therefore of interest to examine the results closely for all deviations from the predicted behaviour.

Sedimentation velocity. The gradient chosen is one in which it was predicted, on the basis of an approximate formula taking into account osmotic effects, that particles having an equilibrium density of 1·22 should sediment at a speed depending only on the angular velocity of the centrifuge and independent of their position. The consequence of this is that the plateau concentration below the boundary should depend only on the fate of the particles which hit the wall of the tube as a result of the radial direction of the centrifugal force. If these particles continue sedimenting at the same rate as the freely moving ones, the plateau concentration should remain unchanged; it should decrease as sedimentation proceeds, if the particles slide down rapidly to the bottom as soon as they have touched the tube wall, and should increase if they trail behind.

Unfortunately, the plateau concentration found in different experiments is not the same, but varies between 0·8 and 1·0 times the initial concentration. This may possibly be due to the presence of some agglutinated material in the preparations or to the co-sedimentation of adsorbed lysosomal enzymes together with the mitochondria. Such artifacts should lower the plateau concentration, and since the latter may in some cases reach almost unity we conclude, in agreement with Kuff, Hogeboom & Dalton (1956), that impact with the side-wall probably has little effect on the rate of sedimentation of the particles. Accordingly, in computing the differentiated curves of Fig. 5 we have introduced no geometrical correction ($\gamma = 0$, de Duve *et al.* 1959), but have considered instead the plateau concentration as representing the 100% base line of the population analysed.

In our opinion, these differentiated curves have some advantages in that they provide a more easily grasped representation of the experimental results and give a direct estimate of the range of sedimentation coefficients or sizes characterizing each particle population. However, too much significance should not be attached to their exact shape, since the boundaries themselves are not known with enough precision to permit accurate differentiation. In addition, it should be stressed that the curves apply to the distribution of enzymic activity and not that of the particles themselves, being directly applicable to the latter only if each particle of a given population contains the same amount of enzyme, a rather improbable assumption.

In design, though not in some of the calculations, our method is similar to that used by Kuff *et al.* (1956) in comparable experiments. In contrast, Thomson and his co-workers (Thomson & Mikuta, 1954; Thomson & Moss, 1956; Thomson & Klipfel, 1957, 1958) have applied another technique, in which the particles are layered above the gradient. The latter technique has the advantage that it furnishes the differentiated pictures as the direct experimental result. However, the thickness of the layer causes an artificial broadening of the bands and the existence of an interface may be responsible for some artifacts, as shown by Anderson (1955).

Density equilibration. Both by their high viscosity and by their very high osmotic pressure, aqueous sucrose solutions are far from ideal in isopycnic experiments. Our principal aim in using heavy water was to reduce the concentration of sucrose required for equilibration. This aim has been largely fulfilled with the mitochondria, but less with the lysosomes and uricase-containing particles owing to an unexpected increase of the equilibrium density of the particles in heavy water. According to de Duve *et al.* (1959), the equilibrium density is theoretically independent of the density of the solvent to the extent that the density of the framework of the particle is itself independent of the density of the solvent. Consequently, the observed deviations from theoretical behaviour are presumably due to the presence of exchangeable bound water of hydration in this framework. The hydration ratio must be particularly high for the uricase-containing particles, for which the peak equilibrium density is 1·228 in water and 1·273 in deuterium oxide. On the other hand, the mitochondria either contain very little bound water or their bound water does not exchange as rapidly. This latter interpretation could explain the second mitochondrial peak which is seen in heavy water and which could be due to particles in which the exchange has taken place, possibly as the result of some damage.

Properties of particles

Wherever comparison is possible, our results are in agreement with those of Thomson and co-workers (Thomson & Mikuta, 1954; Thomson & Moss, 1956; Thomson & Klipfel, 1957, 1958) and of Kuff *et al.* (1956). They fully confirm the distinction between mitochondria and lysosomes and provide additional information about the properties of these particles.

Mitochondria. As shown by the graphs of Figs. 9–11, in which have been included the fractionation results described by Beaufay *et al.* (1959), there is a remarkable correlation between the content of mitochondrial subfractions in glutamic dehydrogenase, malic dehydrogenase or alkaline deoxyribonuclease and their content in cytochrome oxidase. The same correlation exists between cytochrome oxidase and total protein in the fractions separated by gradient centrifuging (Fig. 12), except that in this case the regression lines do not pass through the origin, owing to the existence of an approximately constant amount of extraneous proteins in all subfractions. From Fig. 12 it may be concluded that between 80 and 90% of the proteins of the analysed fractions belonged to mitochondria and that in all the mitochondrial subgroups which were separated in these experiments, the specific activity of cytochrome oxidase, and consequently also of glutamic dehydrogenase, malic dehydrogenase and alkaline deoxyribonuclease, were essentially the same. In view of the numerous factors, size, density, osmotic behaviour, uptake of deuterium oxide, which intervene in determining the distributions observed, such results provide strong additional support to the hypothesis that mitochondria are essentially homogeneous in enzymic content (de Duve *et al.* 1955; de Duve, 1957).

Lysosomes. The five lysosomal hydrolases show typical differences from the mitochondrial group as well as obvious similarities in behaviour, which further confirm the contention that they belong to a distinct group of particles. The enzymic heterogeneity of this group, which contrasts with the homogeneity of the mitochondria, has been repeatedly discussed (de Duve *et al.* 1955; de Duve, 1957; Beaufay & de Duve, 1959) and is again evident in the present experiments. The greatest differences observed are in the ratio of acid phosphatase to acid deoxyribonuclease, which may vary from 1·5 in the less dense fractions to 0·2 in the denser ones.

Obviously, it is possible that such differences reflect the existence of distinct classes of lysosomes, perhaps as many as there are lysosomal enzymes. But it is equally possible that these particles are intrinsically heterogeneous with respect to their enzyme content, for reasons connected, for instance, with their mode of formation and of breakdown within the cells. In this respect, a comparative study of the lysosomes and of the presumably related phagosomes of Straus (1958) should prove very rewarding. A morphological study of the purified sediments which can be isolated in heavy water might also throw some light on the problem.

Uricase-containing particles. Uricase presents another vexing problem which has been discussed before. According to de Duve *et al.* (1955), this enzyme belongs either to the insoluble framework of the lysosomes or to yet another class of particles. With the new evidence available, we can only repeat this statement, with perhaps a slightly greater emphasis on the second possibility. One feature of the uricase-containing particles which has been noted before and stands out clearly in all the present experiments is their relative homogeneity with respect to sedimentation coefficient and density. This is in rather sharp contrast with the heterogeneity of the lysosomes. In addition, the uricase particles sediment somewhat more slowly and are denser, especially in heavy water, than the average lysosomes. Actually, the density-distribution curve of uricase in deuterium oxide–sucrose differs to such an extent from that of acid

phosphatase that there can be little doubt about the actual dissociation of the two enzymes. However, it differs less from that of acid deoxyribonuclease, and the problem of the relationship between uricase and lysosomes must therefore remain open.

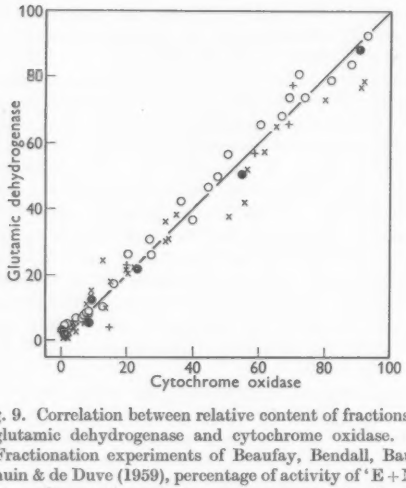


Fig. 9. Correlation between relative content of fractions in glutamic dehydrogenase and cytochrome oxidase. ●, Fractionation experiments of Beaufay, Bendall, Baudhuin & de Duve (1959), percentage of activity of 'E+N'; ○, sedimentation-velocity measurements in density gradient, $(C \times 100)/C_i$; +, density equilibration in water–sucrose, $(C \times 100)/(C_i \times 6)$; ×, density equilibration in D_2O–sucrose, $(C \times 100)/(C_i \times 6)$.

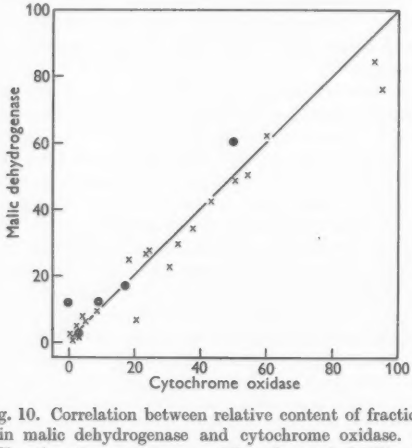


Fig. 10. Correlation between relative content of fractions in malic dehydrogenase and cytochrome oxidase. ●, Fractionation experiments of Beaufay *et al.* (1959), percentage of activity of homogenate ('E+N'); ×, density equilibration in D_2O–sucrose, $(C \times 100)/(C_i \times 6)$.

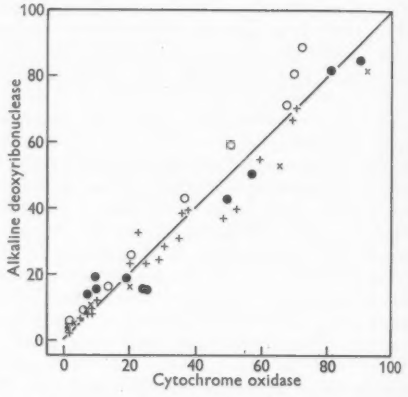


Fig. 11. Correlation between relative content of fractions in alkaline deoxyribonuclease and cytochrome oxidase. ●, Fractionation experiment of Beaufay *et al.* (1959), percentage of activity of homogenate ('E+N'); ○, sedimentation-velocity measurements in density gradient $(C \times 100)/C_i$; +, density equilibration in water–sucrose, $(C \times 100)/(C_i \times 6)$; ×, density equilibration in D_2O–sucrose, $(C \times 100)/(C_i \times 6)$.

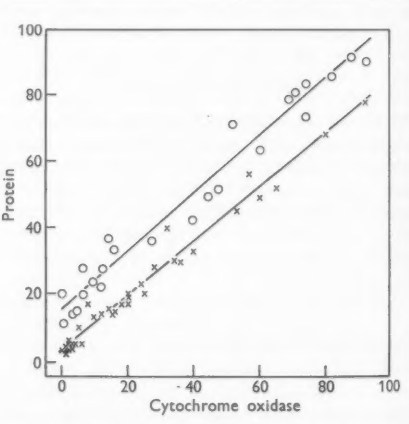


Fig. 12. Correlation between content of fractions in protein and cytochrome oxidase. ○, Sedimentation-velocity measurements in density gradient, $(C \times 100)/C_i$; ×, density equilibration in D_2O–sucrose, $(C \times 100)/(C_i \times 6)$. Regression lines calculated by the method of mean squares were practically the same whether one or the other variable was taken as independent (correlation coefficients were 0·99 and 0·98). Lines drawn are means between the two calculated lines. Note that the upper line (○—○) has co-ordinate values six times larger than the lower line (×—×) and, if drawn on the same scale, would be indistinguishable from the first sixth of the latter.

SUMMARY

1. Mitochondrial fractions from rat liver have been analysed by means of density-gradient centrifuging. Two types of experiments were performed. In one, sedimentation boundaries and the derived sedimentation-coefficient distribution curves were determined after incomplete sedimentation in a stabilizing gradient. In the other, solutions of high density were used to make the gradient and the particles were allowed to approach density equilibrium with the medium by prolonged centrifuging at high speed. In each case, the distributions of up to nine different enzymes were studied simultaneously.

2. Cytochrome oxidase, glutamic dehydrogenase, malic dehydrogenase and alkaline deoxyribonuclease showed practically identical distributions under all conditions applied. The bulk of the proteins of the preparation showed the same distribution pattern, which undoubtedly was that of the true mitochondria. These particles appeared to be remarkably homogeneous with respect to their enzymic content.

3. The five lysosomal hydrolases, acid phosphatase, β-glucuronidase, cathepsin, acid ribonuclease and acid deoxyribonuclease, formed another distinct group, characterized by a lower range of sedimentation coefficients, a greater dispersion of density and a higher average density, especially in heavy water, than the mitochondria. The distribution curves of these enzymes were similar but not identical, indicating that if lysosomes form a single species, they are not enzymically homogeneous.

4. Uricase showed many properties in common with the lysosomal enzymes, but differed from them by having sharper distribution curves and a particularly large increase in equilibrium density in heavy water.

This work has been supported by grants from the Centre National de Recherches sur la Croissance normale et pathologique, the Centre National de Recherches Enzymologiques, the Rockefeller Foundation and the Lilly Research Laboratories. One of us (D.S.B.) is indebted to the Belgian Government for a scholarship and to the Administrators of the H.E. Durham Fund, King's College, Cambridge, for a grant. The authors also acknowledge gratefully the kind help and co-operation of Dr J. Berthet.

REFERENCES

Anderson, N. G. (1955). *Exp. Cell Res.* **9**, 446.
Appelmans, F. & de Duve, C. (1955). *Biochem. J.* **59**, 426.
Appelmans, F., Wattiaux, R. & de Duve, C. (1955). *Biochem. J.* **59**, 438.
Baudhuin, P. (1959). *Arch. int. Physiol. Biochim.* **67**, 106.
Beaufay, H., Baudhuin, P. & Bendall, D. S. (1958). *Abstr. 4th int. Congr. Biochem., Vienna*, p. 71.
Beaufay, H., Bendall, D. S., Baudhuin, P. & de Duve, C. (1959). *Biochem. J.* **73**, 623.
Beaufay, H. & de Duve, C. (1959). *Biochem. J.* **73**, 604.
de Duve, C. (1957). *Symp. Soc. exp. Biol.* **10**, 50.
de Duve, C., Berthet, J. & Beaufay, H. (1959). *Progr. Biophys.* **9**, 325.
de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955). *Biochem. J.* **60**, 604.
Kuff, E. L., Hogeboom, G. H. & Dalton, A. J. (1956). *J. biophys. biochem. Cytol.* **2**, 33.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Straus, W. (1958). *J. biophys. biochem. Cytol.* **4**, 541.
Tedeschi, H. & Harris, D. L. (1955). *Arch. Biochem. Biophys.* **58**, 52.
Thomson, J. F. & Klipfel, F. L. (1957). *Arch. Biochem. Biophys.* **70**, 224.
Thomson, J. F. & Klipfel, F. L. (1958). *Exp. Cell Res.* **14**, 612.
Thomson, J. F. & Mikuta, E. T. (1954). *Arch. Biochem. Biophys.* **51**, 487.
Thomson, J. F. & Moss, E. M. (1956). *Arch. Biochem. Biophys.* **61**, 456.
Wattiaux, R. & de Duve, C. (1956). *Biochem. J.* **63**, 606.

Yield of Oxidative Phosphorylation Associated with the Oxidation of Succinate to Fumarate

By P. GREENGARD,* K. MINNAERT, E. C. SLATER and I. BETEL

Molteno Institute, University of Cambridge and Laboratory of Physiological Chemistry, University of Amsterdam

(*Received* 4 *May* 1959)

Studies of oxidative phosphorylation with succinate as substrate have a special interest, since succinate enters the respiratory chain at a point closer to oxygen than other substrates, and pyridine nucleotide is not involved in the reaction. Unfortunately, since fumarate is readily oxidized by mitochondria which carry out oxidative phosphorylation, it is often difficult to study phosphorylation coupled to the oxidation of succinate to fumarate without complications caused by the further oxidation of the fumarate.

Phosphorylation coupled with the oxidation of succinate to fumarate was first shown by Belitzer & Tsibakowa (1939), who added arsenite to prevent

* Present address: Geigy Research Laboratories, P.O. Box 430, Yonkers, New York, U.S.A.

the oxidation of fumarate (see also Colowick, Welch & Cori, 1940). Green *et al.* (1953) also found some phosphorylation in this reaction, using partially disrupted mitochondria which did not oxidize fumarate. However, either the addition of arsenite or the disruption of mitochondria impairs the phosphorylative activity, and neither treatment can be used to determine the yield of phosphorylation associated with the oxidation of succinate to fumarate by intact mitochondria.

A number of papers have reported P:O ratios approaching or even exceeding 2, with liver mitochondria oxidizing succinate. The most thorough study is that of Copenhaver & Lardy (1952), who found a mean P:O ratio of 1·7. However, it was not possible to determine from this work the proportion of the oxygen uptake associated with the further oxidation of fumarate, and it was therefore not possible to calculate the true P:O ratio for the step succinate → fumarate. Whittam, Bartley & Weber (1955) (see also Krebs, Ruffo, Johnson, Eggleston & Hems, 1953) were able to limit the reaction essentially to this step by using a high concentration of succinate, and found a P:O ratio of 2·4. However, since Whittam *et al.* measured the rate of phosphorylation by determining the rate of incorporation of ^{32}P from inorganic phosphate into adenosine triphosphate, there is a strong possibility that the calculated ratio exceeds the true P:O ratio (Korkes, 1952; Slater & Holton, 1954; Ernster, Ljunggren & Lindberg, 1954; Slater, 1956).

Thus when this investigation began (in 1954), we did not feel that it had been established that the oxidation of succinate to fumarate was accompanied by two phosphorylation steps (see Slater, 1956). Our doubts were influenced by the fact that our preparations of rat-heart sarcosomes (mitochondria), which oxidized fumarate relatively slowly, gave a P:O ratio of about 1 with succinate. During the last five years, however, a number of observations reported in the literature made it increasingly likely that there were, in fact, two phosphorylation steps, and this has become widely accepted. The first convincing evidence was the demonstration by Nielsen & Lehninger (1954), later confirmed by Slater (1954), that phosphorylation occurred in the cytochrome *c* oxidase reaction. Nevertheless it appeared desirable to confirm directly that there were two phosphorylative steps in the oxidation of succinate to fumarate. Experiments carried out for this purpose are described in this paper.

EXPERIMENTAL

Materials

Chemicals and enzymes used in measurements of oxidative phosphorlyation were the same as described in previous papers (Slater, 1953; Slater & Holton, 1954). In some experiments, adenosine diphosphate (ADP) manufactured by the Sigma Chemical Co. (St Louis, Mo., U.S.A.) was used.

Guanosine diphosphate (GDP), cytidine diphosphate (CDP), uridine diphosphate (UDP), adenosine triphosphate (ATP) and adenosine monophosphate (AMP) were obtained from the Sigma Chemical Co.

Tissue preparations

Liver mitochondria. Mitochondria were isolated from the livers of male Wistar rats by the method of Hogeboom (1955), slightly modified as described by Myers & Slater (1957).

Heart sarcosomes (mitochondria). Two types of preparations were used. (1) Sarcosomes isolated from rat heart in 0·21 M-sucrose–10 mM-ethylenediaminetetra-acetate (EDTA), pH 7·4, by the method of Cleland & Slater (1953), and suspended in the same medium. These will be referred to as CS sarcosomes. (2) Sarcosomes isolated from mice, rat or guinea-pig hearts essentially according to the procedure of Holton, Hülsmann, Myers & Slater (1957).

Each preparation was made from the heart of one rat (male), or one guinea pig (male) or from six mouse hearts. The animal was killed by a blow on the head and the heart quickly removed. Auricular tissue and connective tissue and fat were removed and the heart was then placed in an ice-cold solution of 0·23 M-sucrose–5 mM-ATP, pH 7·4. The solution was decanted and replaced by fresh solution. The heart was finely minced with scissors and, in two to three batches, was homogenized for 10–15 sec. at moderately high speed in a Potter–Elvehjem homogenizer fitted with a polytetrafluoroethylene (Teflon) pestle and containing 5–10 ml. of the sucrose–ATP soln. The homogenizer tube was immersed in ice–water during this operation. The homogenate from each batch of heart was poured into cold centrifuge tubes and the combined homogenates were centrifuged for 3 min. at 600*g* at 0–2°. The supernatant was decanted into other centrifuge tubes, without contamination by the sediment, and centrifuged for 15 min. at 600*g* at 0–2°. The supernatant was completely decanted and the sediment suspended in an ice-cold solution of 0·24 M-sucrose–1 mM-ATP, pH 7·4 with the help of the homogenizer (hand-operated). The concentration of the sarcosomes in this suspension was usually about 3–10 mg. of sarcosomal protein/ml. This preparation will be referred to as HHMS sarcosomes.

Liver mitochondrial extract. This was prepared according to the method of Dianzani (1956).

Methods

Adenosine triphosphatase. Adenosine triphosphatase activity of isolated mitochondria or sarcosomes was determined at room temperature (18–22°) as described by Myers & Slater (1957), in the following reaction mixture: ATP, pH 7·4, 2 mM; KCl, 75 mM; EDTA, pH 7·4, 1 mM; $MgCl_2$, 3 mM; sucrose, 10 mM, with or without 0·1 mM-2:4-dinitrophenol. The stimulation of the adenosine triphosphatase by dinitrophenol is expressed by the ratio, *r*, which equals (activity in the presence of dinitrophenol)/(activity in the absence of dinitrophenol).

P:O *ratio.* The method with oxygen as acceptor was the same as that described by Slater & Holton (1954). Differential manometers, with 6-ml. flasks, fluid vol. 1 ml., were used. Manometer constants were calculated according

to van Dorp & Slater (1959). The O_2 uptake during the equilibration period was determined by extrapolation as described by Slater & Holton (1954). This sometimes caused some difficulty, because of a rapid decline from the initial rate of O_2 uptake. More uniform rates were obtained in the presence of NaF and high concentrations of succinate. The standard reaction mixture contained potassium phosphate, pH 7·4, 30 mM; glucose, 30 mM; ADP, 0·6 mM; AMP, 0·6 mM; NaF, 20 mM; $MgCl_2$, 5 mM; EDTA, 2 mM; cytochrome *c*, 20 μM; disodium succinate, 60 mM; hexokinase. The reaction mixture also contained 50 mM-sucrose introduced with the suspension of liver mitochondria, 20 mM-sucrose introduced with the suspension of CS sarcosomes or 20 mM-sucrose–0·1 mM-ATP introduced with the suspension of the HHMS sarcosomes. The reaction period was usually between 20 and 35 min. at 25°; it was found with heart sarcosomes (CS) that the P:O ratio was constant for at least 60 min. (Fig. 1). Respiratory activity is expressed as Q_{O_2} (μl. of O_2/mg. of mitochondrial protein/hr.); protein was determined by the biuret method (see Cleland & Slater, 1953).

The method with ferricytochrome *c* as acceptor was that described on p. 394 of Slater (1955) under the subheading '(iii) Spectrophotometric Thunberg tubes'. The reaction mixture contained potassium phosphate, pH 7·4, 30 mM; ADP, 0·19 mM; AMP, 0·19 mM; NaF, 20–40 mM; disodium succinate, 60 mM; glucose, 30 mM; $MgCl_2$, 5 mM; EDTA, 1 mM; hexokinase; sarcosomes (CS), 0·2–0·25 mg. of protein/ml.; 0·573 μg.atom O equiv. of ferricytochrome *c*. The reduction of the cytochrome *c*, which was followed by measuring the decrease of the extinction at 580 mμ, was complete in 11–20 min. at room temperature. The amount of ferricytochrome *c* reduced, calculated from $\Delta E_{580\,m\mu}$, was 0·56–0·58 μg.-atom O equiv., practically identical with the amount added.

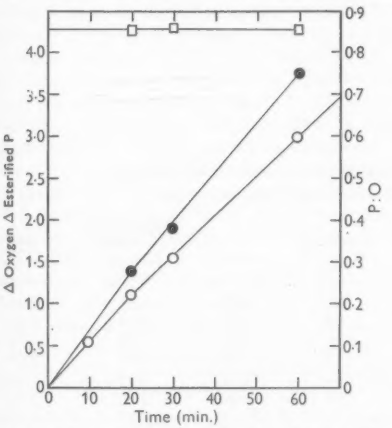


Fig. 1. Stability of oxidative phosphorylation with rat-heart sarcosomes (CS). The standard reaction mixture (see under Methods) was used, with 0·37 mg. of sarcosomal protein/ml. and 366 units (Berger, Slein, Colowick & Cori, 1946) of hexokinase/mg. of sarcosomal protein. ●, Oxygen uptake (μg.atoms); ○, Δ esterified P (Δ hexose monophosphate + Δ~P; see Slater & Holton, 1954); □, P:O ratio.

Malic dehydrogenase. In order to measure the activity of the pyridine nucleotide dehydrogenases in mitochondria, it is necessary to subject the latter to a preliminary hypo-osmotic treatment so that the pyridine nucleotide is readily accessible to the dehydrogenase (Christie & Judah, 1953). The mitochondria or sarcosomes were suspended in water at 0° for 3–5 hr. and, after brief treatment in a Potter–Elvehjem homogenizer in order to damage the mitochondria further and thereby facilitate the accessibility of the pyridine nucleotide to the dehydrogenase, a sample of the mitochondrial suspension was added to a spectrophotometric cuvette at 25° containing 80 μM-reduced diphosphopyridine nucleotide (DPNH), prepared as in Slater (1953), 30 mM-2-amino-2-hydroxymethylpropane-1:3-diol–HCl buffer, pH 7·4; 3% of ethanol (derived from the DPNH and antimycin solutions) and 130–180 μg. of antimycin/mg. of protein. (The antimycin was included to inhibit the DPNH oxidase system.) Measurements at 340 mμ were taken to confirm that no oxidation of DPNH occurred and then, 5 min. after the addition of the mitochondrial suspension, dipotassium oxaloacetate was added to a final concentration of 0·39 mM, and the rate of decrease of the extinction at 340 mμ was measured. Doubling the DPNH concentration had no effect on the activity. One unit of malic dehydrogenase is defined as the amount which caused a decrease of extinction of 0·01/min. at 25°, and specific activity as the number of units/mg. of protein in the reaction mixture.

Fumarate plus malate. The amount of (fumarate plus malate) present after oxidation of succinate by mitochondria or sarcosomes was determined by means of malic decarboxylase (Korkes, del Campillo & Ochoa, 1950; Nossal, 1951, 1952). In these experiments, oxidation of succinate was carried out according to the procedure and with the same reaction mixture as described above for measurements of the P:O ratio, except that two concentrations of succinate (6·6 mM and 66 mM) were used and the reaction volume was 1·5 ml. After oxidation for 20–30 min., the reaction was stopped by the addition of 0·1 ml. of 10% (w/v) acetic acid, and the contents of the manometer flask were transferred to a centrifuge tube, which was placed in a boiling-water bath for 2 min. After cooling, the precipitated protein was removed by centrifuging, and the clear supernatant (pH 5·0) was used for the estimation of the (fumarate + malate) content.

A sample (0·9 ml.) of this supernatant was added to the main compartment of a second 6 ml. manometer flask, which contained 15 units of fumarase [prepared from pig heart according to the method of Massey (1955) up to a specific activity of 27·5 units/mg. of protein], 2·1 μmoles of glucose, 1·87 μmoles of $MnCl_2$, 75 μmoles of KCl, 18·5 μmoles of sodium acetate, pH 5·0, and 1 μmole of aniline. The side arm of the flask contained 2·1 μmoles of glucose, 1·87 μmoles of $MnCl_2$, 75 μmoles of KCl, 18·5 μmoles of sodium acetate, pH 5·0, and 0·1–0·2 ml. of a washed-cell suspension (20%, w/v) of malate-adapted *Lactobacillus arabinosus* (Nossal, 1951, 1952) in 0·1 M-KCl. The total fluid volume in the manometer flask was 1·5 ml. The gas phase was air. The amount of CO_2 liberated from the (malate + fumarate) was measured at 37°. The reaction was complete in 20–30 min.

For testing this method, the Keilin & Hartree heart-muscle preparation (Slater, 1949), which does not oxidize

malate, was used to oxidize the succinate. The reaction was continued until all the succinate was oxidized (Expt. 1, Table 1). There is close agreement between the amount of succinate used, the O_2 uptake and the amount of (fumarate + malate) found at the end of the reaction. In Expt. 2, in which the succinate was not completely oxidized, there was a good agreement between the O_2 uptake and the amount of fumarate + malate found.

RESULTS

Effects of change of the reaction mixture on the P:O *ratio*

Succinate concentration. The effects of varying the succinate concentration between 3 and 60 mM are shown in Table 2. The Q_{O_2} increased with increasing succinate concentration within this range, even though this was far above that normally necessary to saturate succinic dehydrogenase.

With liver mitochondria, the P:O ratio decreased with increasing succinate concentration whereas with heart sarcosomes variation of the succinate concentration between 6 and 60 mM had no effect on the ratio.

Table 3 shows that with 6·6 mM-succinate and liver mitochondria, the O_2 uptake is 1·66–1·83 times the amount of (fumarate + malate) found, showing that under these conditions oxidation proceeds past fumarate. With higher concentrations of succinate (66 mM), however, the O_2 uptake exceeded the (fumarate + malate) found by only 3–10 %. With heart sarcosomes, there was little oxidation past fumarate, even with the lower concentrations of succinate.

Table 1. *Test of method of determining fumarate plus malate*

Heart-muscle preparation, 5 mg. of protein in Expt. 1, 0·5 mg. in Expt. 2; phosphate, pH 7·4, 40 mM; EDTA, 10 mM; vol., 1·5 ml. In Expt. 1 the reaction was run until all the succinate was oxidized (43 min.). In Expt. 2 the reaction was stopped after 17 min.

Expt. no.	Initial amount of succinate (μmoles)	O_2 uptake (μg.atoms)	Fumarate + malate at end of reaction (μmoles)
1	7·0	7·2	7·05
2	50·0	7·55	7·34

Table 2. *Effect of succinate concentration of* P:O *ratio*

Values given are the means of a number of experiments, in which the concentrations of succinate were varied.

	Concn. of succinate (mM)				
	3	6	15	30	60
Rat-liver mitochondria					
No. of expts.	3	6	2	1	9
Q_{O_2}	15	23	34	56	109
P:O	2·20	2·17	2·14	1·94	1·62
Rat-heart sarcosomes (CS)					
No. of expts.		5	3	3	6
Q_{O_2} (mean)		43	56	66	165
P:O (mean)		0·95	0·93	0·90	0·93

Table 3. *Relationship between oxygen consumed and (fumarate plus malate) formed by rat-liver and -heart mitochondria oxidizing succinate*

Expt. no.	Mitochondria	Protein (mg.)	Time of expt. (min.)	Concn. of succinate (mM)	Concn. of Amytal (mM)	ΔO (μg.atoms)	Δ (fumarate + malate) formed (μmoles)	ΔO / Δ (fumarate + malate)
1	Liver	2·1	29·5	6·6	—	5·83	3·20	1·83
			31	6·6	1·8	6·49	6·24	1·04
			26·5	66	—	7·70	6·95	1·10
			28·5	66	1·8	7·45	7·35	1·01
2	Liver	1·6	32·5	6·6	—	1·64	0·99	1·66
			33	6·6	1·8	1·49	1·40	1·06
			30	66	—	4·80	4·67	1·03
			30·5	66	1·8	4·35	4·35	1·00
3	Heart (HHMS)	0·7	26·5	6·6	—	1·00	0·98	1·02
			26·5	6·6	1·8	1·60	1·65	0·97
			22·5	66	—	2·90	2·94	0·99
			25	66	1·8	4·78	4·99	0·96
4	Heart (HHMS)	0·57	31	6·6	—	1·02	0·98	1·04
			32	6·6	1·8	2·14	1·97	1·09
			27·5	66	—	3·12	3·11	1·00
			28	66	1·8	4·98	4·59	1·09

Table 4. *Effect of Amytal on* P:O *ratio with liver mitochondria*

Means of three expts., each in duplicate.

Concn. of succinate (mM)	P:O		
	Without Amytal	With 1·8 mM-Amytal	Δ
6	1·92	1·36	−0·56
60	1·44	1·28	−0·16
Δ	−0·48	−0·08	—

Table 5. *Malic dehydrogenase activity of rat-liver mitochondria and rat-heart sarcosomes*

Malic dehydrogenase activity was measured as described under Methods. The succinic oxidase activity of the intact mitochondria or sarcosomes was measured with the standard reaction mixture.

	Mitochondria	Sarcosomes (HHMS)
Malic dehydrogenase specific activity (*a*)	870	350
Succinic oxidase Q_{O_2} (*b*)	85	105
(*a*)/(*b*)	10·2	3·3

Attempts to inhibit the oxidation of malate by the acetylpyridine or pyridine-3-aldehyde analogues of diphosphopyridine nucleotide (DPN), kindly supplied by Dr N. O. Kaplan, were unsuccessful. These compounds slightly stimulated the oxidation of malate by liver mitochondria, held for 3·5 hr. at 0° in order to increase the permeability of the mitochondria to these compounds.

Jalling, Lindberg & Ernster (1955) found that 1·8 mM-Amytal (5-amino-5-ethylbarbituric acid) completely inhibited the oxidation by liver mitochondria of substrates dependent upon DPN, without affecting the oxidation of succinate. In agreement with these findings, added malate was not oxidized by our preparations of liver mitochondria in the presence of Amytal of this concentration, and Table 3 shows that the addition of Amytal caused a decrease of the ratio $\Delta O/\Delta$(fumarate + malate) from 1·83 and 1·66 to 1·04 and 1·06 respectively.

From these results it appears likely that the higher P:O ratio obtained with liver mitochondria at low succinate concentrations is due to the fact that under these conditions a considerable proportion of the O_2 uptake is associated with the oxidation of malate, which has a higher P:O ratio. This interpretation is supported by the results given in Table 4, which show that in the presence of Amytal, the P:O ratio becomes largely independent of the concentration of succinate, and that Amytal has much less effect on the P:O ratio obtained with 60 mM-succinate than with 6 mM-succinate.

Even with low concentrations of succinate, there is little oxidation past fumarate–malate with heart sarcosomes (Table 3). A partial explanation is the relatively lower malic dehydrogenase activity of the sarcosomes (Table 5).

Concentration of mitochondria or sarcosomes. Variation of the concentration of liver mitochondria between 0·4 and 6·65 mg. of protein/ml. or of the concentration of heart sarcosomes (HHMS) between 0·16 and 1·7 mg. of protein/ml. had no appreciable effect on the Q_{O_2} or on the P:O ratio. The addition of serum albumin (5 mg./ml.) also had no effect.

Hexokinase concentration. The P:O ratio with liver mitochondria was unaffected by varying the concentration of hexokinase between 10 and 600 units (Berger, Slein, Colowick & Cori, 1946)/mg. of mitochondrial protein. With heart sarcosomes (CS), in the absence of fluoride, the following values were obtained with different concentrations of hexokinase: 40 units/mg. of protein, 0·68; 120 units/mg. of protein, 0·90; 400 units/mg. of protein, 0·88; 800 units/mg. of protein, 0·95; extrapolated to infinite hexokinase concentration, 0·96. The value for infinite hexokinase concentration was calculated from the straight line obtained by plotting 1/P:O against 1/[hexokinase] (see Lewis & Slater, 1954). It can also be calculated from this straight line that, in the absence of fluoride, 400 units of hexokinase/mg. of protein (the usual concentration with the CS sarcosomes) underestimates by only 3% the P:O ratio obtained with an infinite concentration of hexokinase.

Ethylenediaminetetra-acetate. When purified reagents were used, the addition of 2 mM-EDTA had no effect on the Q_{O_2} or on the P:O ratio with liver mitochondria oxidizing α-oxoglutarate, succinate, glutamate or β-hydroxybutyrate. The effect of EDTA on heart sarcosomes has been reported by Slater & Cleland (1953).

Magnesium and phosphate. Variation of the concentration of magnesium and phosphate, with 2 mM-EDTA and 20 mM-NaF, showed that the highest P:O ratios with heart sarcosomes were obtained with 5 mM-magnesium and 30 mM-phosphate. However, a considerable departure from the optimum conditions had only a small effect on the P:O ratio.

pH. The effect of pH on the P:O ratio with heart sarcosomes has been studied by Hülsmann & Slater (unpublished work). The maximum P:O ratio, which was found at 6·5, was in the experiments of these authors 19% (guinea pig) and 16% (rat) greater than that at 7·4, the pH used in the present study.

Fluoride. Table 6 shows that fluoride up to 0·04M had no effect on the P:O ratio with heart sarcosomes, in agreement with Slater & Bonner (1952). Its effect on liver mitochondria was variable. Fluoride increased the P:O ratio with both high and low succinate concentrations, in Table 6, Expt. 1. Since at the same time the Q_{O_2} was lowered, this

result could be explained by the fact that fluoride, in the presence of phosphate, is a competitive inhibitor of succinate dehydrogenase (Slater & Bonner, 1952). Thus fluoride might be expected to have the same effect as decreasing the succinate concentration. On the other hand, in Expt. 2, Table 6, fluoride had little effect on the P:O ratio, although it markedly decreased the Q_{O_2}.

These results give no support to the suggestion (Whittam *et al.* 1955) that fluoride, in the concentrations often used in studying oxidative phosphorylation, might lower the P:O ratio.

Cytochrome c. Variation of the cytochrome *c* concentration between 0 and 0·1 mM had no effect on the P:O ratio with heart sarcosomes. However, the Q_{O_2} is in some preparations slightly enhanced by the addition of cytochrome *c*. A concentration of 2 μM-cytochrome *c* was sufficient for the maximum stimulation. This is much less than is required by the succinic oxidase system of the Keilin & Hartree heart-muscle preparation (sarcosomal fragments) (see Slater, 1949).

Adenosine diphosphate. Variation of the ADP concentration between 0·1 and 1·2 mM had no effect on the ratio with rat-heart sarcosomes (CS).

Addition of possible cofactors. Neither the P:O ratio nor the Q_{O_2} was affected by the addition of UDP, CDP or GDP, alone or in combination with ADP. Similarly, no effect was found by adding an extract of liver mitochondria prepared according to the method of Dianzani (1956), who found that the P:O ratio of aged liver mitochondria was increased by the addition of this extract.

The effect of adding Mn^{2+} ions was also studied, in view of the finding by Ernster, Lindberg & Löw (1955) that this addition decreased the amount of ATP required to restore phosphorylation associated with the oxidation of succinate after a pre-incubation of liver mitochondria with Ca^{2+} ions. Table 7 shows that Mn^{2+} ions did not increase the P:O ratio with rat-heart sarcosomes. Low concentrations slightly increased the Q_{O_2}, without affecting the P:O ratio, whereas higher concentrations inhibited both the oxidation and the phosphorylation.

Table 6. *Effect of fluoride*

The P:O values for heart sarcosomes are the means of five experiments. The Q_{O_2} values for heart sarcosomes are means of five to eight experiments. The two experiments with liver are each single experiments.

Preparation	Concn. of succinate (mM)	Concn. of fluoride (mM)	Q_{O_2}	P:O
Liver (Expt. 1)	6	0	48·5	1·91
	6	0·02	24	2·31
	60	0	154	1·37
	60	0·02	83	1·94
Liver (Expt. 2)	6	0	85	2·03
	6	0·02	31	1·95
	60	0	177	1·43
	60	0·02	135	1·47
Heart (CS)	60	0	317	1·01
	60	0·01	—	1·09
	60	0·02	182	1·04
	60	0·04	121	1·08
	60	0·08	—	0·59

P:O *ratio for oxidation of succinate to fumarate*

The mean values of different preparations are shown in Table 8. In our hands, isolated heart sarcosomes have appreciably lower P:O ratios than isolated liver mitochondria. They also have a higher adenosine triphosphatase activity in the presence of Mg^{2+} ions and a lower degree of stimulation of the adenosine triphosphatase by DNP. The isolation medium used in preparing the HHMS sarcosomes in this paper was selected out of 15 media tried by Holton *et al.* (1957) as giving the greatest stimulation of adenosine triphosphatase by dinitrophenol. The highest P:O ratio and the greatest degree of stimulation by dinitrophenol was

Table 7. *Effect of manganese*

CS rat-heart sarcosomes were used in Expt. 1; in Expt. 2, the CS rat-heart sarcosomes were washed with and suspended in 0·25 M-sucrose to remove the EDTA. 20 mM-Succinate was used instead of the standard 60 mM-succinate. (Free Mn) = (Total Mn) − (EDTA).

Expt. no.	EDTA (mM)	Mn Total (mM)	Mn Free (mM)	ΔO	Δ Esterified P	P:O
1	1	0	0	4·40	3·42	0·80
	1	1	0	3·78	2·74	0·72
	1	1·5	0·5	3·28	2·35	0·71
	1	2	1	3·17	1·96	0·62
2	0	0	0	4·24	2·68	0·63
	2	0	0	4·81	3·00	0·62
	0	0·05	0·05	4·69	2·92	0·63
	0	0·1	0·1	3·74	2·25	0·60
	0	1	1	3·79	1·86	0·49
	2	1	0	4·24	—	—

Table 8. P:O *ratios of different mitochondrial preparations*

Preparations	No. of determinations	P:O (mean ± S.E.M.)
Rat-heart sarcosomes (CS)	19	1·01 ± 0·04
Rat-heart sarcosomes (HHMS)	14	0·94 ± 0·05
Mouse-heart sarcosomes (HHMS)	1	0·99
Guinea-pig sarcosomes (HHMS)	10	1·31 ± 0·08
Rat-liver mitochondria	12	1·57 ± 0·07

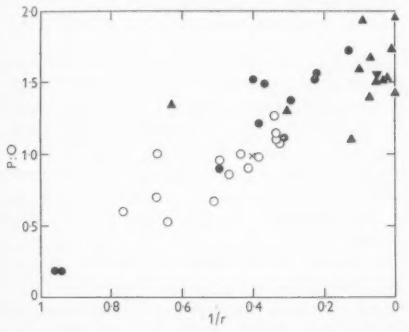


Fig. 2. Relationship between the P:O ratio and the adenosine triphosphatase activity (expressed as $1/r$) of different preparations. This graph includes all our preparations on which both measurements were made, including a few in which the isolation procedure was purposely altered to give damaged mitochondria. ○, Rat-heart sarcosomes (HHMS); ●, guinea-pig-heart sarcosomes (HHMS); ×, mouse-heart sarcosomes; ▲, ▼, rat-liver mitochondria.

found with some preparations isolated from the hearts of young guinea pigs by the method of Holton *et al.* (1957).

The quantitative relationship between the adenosine triphosphatase activity and the P:O ratio of various preparations of heart sarcosomes and liver mitochondria is shown in Fig. 2. The adenosine triphosphatase activity is expressed as $1/r$ (see under Methods), which is a measure of the fraction of the potential adenosine triphosphatase which is active in the absence of dinitrophenol. It is significant that all values, both for liver and heart, lie around the same curve.

Phosphorylation coupled with the reduction of ferricytochrome c *by succinate*

Table 9 summarizes an experiment demonstrating phosphorylation coupled with the reduction of ferricytochrome *c* by succinate. The P:O ratio is small (0·2), but well outside the experimental error. Other experiments are summarized in Table 10. Hypo-osmotic pretreatment of the sarcosomes (CS), which causes increased phosphorylation with ferrocytochrome c as hydrogen donor, brought

Table 9. *Phosphorylation coupled with reduction of ferricytochrome* c *by succinate*

Rat-heart sarcosomes (CS). Phosphorylation was measured (method II) as described by Slater (1955). HMP, Hexose monophosphate; ~P, 2 ATP + ADP (see Slater, 1953).

Ferricytochrome *c* (μg.atoms O equiv.)		0·573	0·573
~P (μmole)	initial	0·727	0·727
	final	0·638	0·661
	Δ	−0·089	−0·066
HMP (μmole)	initial	0·047	0·047
	final	0·225	0·231
	Δ	0·178	0·184
Δ HMP + Δ~P		0·09	0·12
P:O		0·16	0·21

Table 10. *Comparison of phosphorylation ratios with succinate as hydrogen donor and either oxygen or ferricytochrome* c *as hydrogen acceptor*

Normal preparation refers to rat-heart sarcosomes isolated in 0·21 M-sucrose–10 mM-EDTA, pH 7·4. Hypo-osmotic preparation refers to sarcosomes treated as in Table 9 of Slater (1955). Method II (see Slater, 1955) was used to measure phosphorylation in Expt. 1 (see Table 9); method I was used in Expts. 2 and 3.

		P:O	
Expt. no.	Preparation	With O_2	With ferricytochrome *c*
1	Normal CS	0·99	0·18
2	Normal CS	1·11	0·21
3	Normal CS	1·44	0·20
	Means	**1·18**	**0·20**
	Hypo-osmotic CS	—	0·11

about a decrease of the phosphorylation with ferricytochrome *c* as acceptor (cf. Slater, 1955).

In Expt. 2 of Table 10, a control was carried out with fumarate, in a concentration equivalent to that of the ferricytochrome *c* concentration, in the place of the succinate. Only 0·06 μmole of ferricytochrome *c* (0·03 μg.atom O equiv.) was reduced (calc. from $\Delta E_{580\,m\mu}$), with negligible phosphorylation. It is clear that under the conditions of the experiments with ferricytochrome *c* as acceptor and succinate as donor, virtually all the phosphorylation is associated with the reduction of ferricytochrome *c* by succinate.

The P:O ratio with ferrocytochrome *c* as substrate and unswollen rat-heart sarcosomes is 0·12 (Slater, 1956). The experimental results with different donors and acceptors are given as figures above the arrows in the following reaction schemes:

$$\text{Succinate} \xrightarrow{0\cdot2} \text{cyt. } c \xrightarrow{0\cdot12} O_2$$
$$\text{Succinate} \xrightarrow{\qquad} \text{cyt. } c \xrightarrow{\qquad} O_2 \quad (1\cdot0)$$

This illustrates the relative inefficiency of added ferricytochrome *c* as an acceptor or of added ferrocytochrome *c* as a donor.

DISCUSSION

Succinate is often used in studies of oxidative phosphorylation in order to bypass that phosphorylation step in the respiratory chain which involves DPN. Since the further oxidation of fumarate, the product of oxidation of succinate, involves DPN, it is important to select conditions under which the further oxidation does not proceed to any appreciable extent. Table 3 shows that these conditions are not fulfilled with liver mitochondria oxidizing 6 mM-succinate, the concentration mostly used in studies reported in the biochemical literature. The further oxidation of fumarate by liver mitochondria can be made negligible by the addition of Amytal (1·8 mM), or by increasing the succinate concentration to 60 mM. With heart sarcosomes, the further oxidation of fumarate is not appreciable even with low concentrations of succinate.

The effect of increasing the concentration of succinate from 6 to 60 mM on the proportion of the total oxygen uptake which is associated with the further oxidation of the fumarate can be understood by a consideration of the Q_{O_2} values given in Table 2. The Q_{O_2} of liver mitochondria with 60 mM-succinate was 4·75 times that with 6 mM-succinate, and the relative rate of the step succinate → fumarate must have been even greater, since oxidation past fumarate was greater with the lower concentration of succinate (Table 3). This shows that the K_m for succinate in mitochondria is much larger than that found in succinic oxidase preparations, e.g. 0·5 mM found by Slater & Bonner (1952) for the Keilin & Hartree heart-muscle preparation. This suggests that mitochondria contain a competitive inhibitor of succinic dehydrogenase. The effect of increasing the succinate concentration ten-fold will be to increase the velocity of the succinate → fumarate step relative to that of the further oxidation of fumarate, even if the latter reaction remains unaffected. [In fact, it is not unlikely that an increased rate of oxidation of succinate through the respiratory chain would cause an inhibition of the oxidation of other substrates; cf. the inhibition by succinate of DPNH oxidation, studied by Wu & Tsou (1955).]

For these reasons it seems likely that the P:O ratios obtained with 60 mM-succinate are more representative of the step succinate → fumarate than those obtained with lower succinate concentrations. Packer (1958) has suggested that succinate might cause loss of ATP by the reaction

$$\text{ATP} + \text{succinate} + \text{coenzyme A} \rightleftharpoons \text{succinyl-coenzyme A} + \text{ADP} + H_3PO_4$$

This reaction cannot be operating in our experiments, since the hexokinase which was present in great excess would trap the ATP formed. That the higher concentrations of succinate do not have an uncoupling effect is shown by the lack of effect of increasing the succinate concentration to 60 mM on the P:O ratio of heart sarcosomes (Table 2) and of liver mitochondria in the presence of Amytal (Table 4).

The competitive inhibitor of succinic dehydrogenase formed in mitochondria has not been positively identified in this study, but it appears rather likely that it is oxaloacetate. This conclusion is not necessarily in conflict with the fact that succinic dehydrogenase is inhibited even in heart sarcosomes where only a small proportion of the oxygen uptake is associated with the further oxidation of fumarate, since only a very small amount of oxaloacetate is necessary to inhibit succinic dehydrogenase (K_i 1·5 μM). For example, in the presence of 6 mM-succinate, only 70 μM-oxaloacetate is necessary to inhibit succinic dehydrogenase by 80 %. The same concentration of oxaloacetate would inhibit succinic dehydrogenase by 29 % in the presence of 60 mM-succinate. If the inhibitor in mitochondria is oxaloacetate, the degree of inhibition will be determined by the steady-state concentration of oxaloacetate, which will depend upon the rate of decomposition of oxaloacetate as well as upon the rate of its formation, and will not necessarily bear a simple relationship to the extent of oxidation past fumarate. However, quantitative measurements of the small concentrations of oxaloacetate expected are required before it can be accepted with certainty that this is the major competitive inhibitor in mitochondria.

The P:O ratios for rat-liver and heart sarcosomes oxidizing succinate found in this study are in general agreement with most reports in the literature. The ratio with 6 mM-succinate and liver mitochondria which we find (2·17) is not lower than the value (1·70) found by Copenhaver & Lardy (1952) with the same concentration of succinate. Our results for rat-heart sarcosomes (0·98 for all preparations in Table 8) are similar to the value of 1·1 recently reported by Packer (1958). Both our results and those of Packer are considerably lower than that

(1·64) reported by Maley & Plaut (1953, 1954) for rat-heart sarcosomes. The cause of this discrepancy does not lie in the composition of the reaction mixture used by Maley & Plaut, which gave the same P:O ratio as our standard reaction mixture with our sarcosome preparation.

The larger difference between the P:O values with succinate of rat-liver and rat-heart mitochondria reported in the literature is explained by our experiments. With 6 mM-succinate, which is about the concentration used in most studies, the average P:O ratios in Table 2 are 2·17 for liver and 0·95 for heart. About one-half of this difference is due to the fact that under these conditions a considerable proportion of the oxygen uptake with liver mitochondria is associated with the oxidation of fumarate, which yields a higher P:O ratio. This has the effect of exaggerating the true difference between the two types of mitochondria, which we believe is better reflected by the ratios obtained with 60 mM-succinate.

The remaining difference (1·57 with rat-liver mitochondria, 0·98 with rat-heart sarcosomes; see Table 8) is due to the fact that in the latter preparation hydrolytic side reactions, at pH 7·4 in the presence of Mg^{2+} ions, compete with inorganic phosphate for the energy-rich intermediates of oxidative phosphorylation. A quantitative measure of these side reactions is given by $1/r$, where r is the ratio of the adenosine triphosphatase activity in the presence and absence of the uncoupling agent dinitrophenol (Mg^{2+} ions present in both cases). Fig. 2 shows a negative correlation between the P:O ratios of individual preparations of mitochondria (from rat liver and rat, mouse and guinea-pig heart) and the values of $1/r$.

Heart sarcosomes isolated from the guinea pig gave appreciably higher ratios (mean 1·31) than those obtained from the rat, and this difference is correlated with a lower activity of the side reactions in the guinea-pig preparation. Hülsmann & Slater (unpublished work) have found a mean P:O ratio of 1·47 with guinea-pig-heart sarcosomes at pH 6·5. Hatefi & Lester (1958) have reported a P:O ratio of 1·93 with 'heavy' mitochondria isolated from ox heart obtained from a slaughterhouse. They suggest that the lower P:O ratios reported by Cleland & Slater (1953) might be due to the presence of both light and heavy particles in our sarcosomal preparation. However, this does not seem a likely explanation of our present results, since the sarcosomes were isolated by sedimentation at relatively low speed (15 min. at 600 *g*).

Thus there appears to be no fundamental difference between the mechanism of oxidative phosphorylation in liver and heart mitochondria. A P:O ratio substantially above 1·0 for the step succinate → fumarate in rat-liver and guinea-pig-heart mitochondria shows that there are two phosphorylating steps in this reaction in these preparations. There is evidence that this is also the case even with our preparations of rat-heart sarcosomes in which the P:O ratio does not exceed 1·0. With this preparation, phosphorylation has been demonstrated between substrate and ferricytochrome *c* (this paper) and between ferrocytochrome *c* and oxygen (Slater, 1954). Further evidence is provided by a study of the pH–activity curve of the P:O ratio (Hülsmann & Slater, 1957).

SUMMARY

1. Factors affecting the P:O ratio with succinate as substrate were studied, both with rat-liver mitochondria and heart sarcosomes of the rat, mouse and guinea pig. The concentrations of all components of the reaction mixture except succinate could be varied widely, without appreciable effect.

2. Higher P:O ratios were obtained with liver mitochondria with low concentrations of succinate. It is believed that this is because, under these conditions, a large proportion of the total oxygen uptake is associated with oxidation of fumarate, a reaction which gives a higher yield of oxidative phosphorylation. With higher concentrations of succinate there was no appreciable oxidation of the fumarate formed. With heart sarcosomes there was no appreciable oxidation of the fumarate, with either high or low concentrations of succinate.

3. Our preparations of rat-heart sarcosomes gave appreciably lower P:O ratios than liver mitochondria. The lower ratios with sarcosomes are quantitatively correlated with the higher adenosine triphosphatase activities (in the presence of Mg^{2+} ions) of these preparations.

4. Oxidative phosphorylation (with low ratios) was detected, coupled with the reduction of ferricytochrome *c* by succinate.

5. The results support the view generally held that the oxidation of succinate to fumarate by mitochondrial preparations is accompanied by two phosphorylative steps per pair of electrons, even in preparations of rat-heart sarcosomes which give a P:O ratio of 1·0.

REFERENCES

Belitzer, V. A. & Tsibakowa, E. T. (1939). *Biokhimiya*, **4**, 516.

Berger, L., Slein, M. W., Colowick, S. P. & Cori, C. F. (1946). *J. gen. Physiol.* **29**, 379.

Christie, G. S. & Judah, J. D. (1953). *Proc. Roy. Soc.* B, **141**, 420.

Cleland, K. W. & Slater, E. C. (1953). *Biochem. J.* **53**, 547.

Colowick, S. P., Welch, M. S. & Cori, C. F. (1940). *J. biol. Chem.* **133**, 359.

Copenhaver, J. H. & Lardy, H. A. (1952). *J. biol. Chem.* **195**, 225.

Dianzani, M. U. (1956). *Biochim. biophys. Acta*, **22**, 389.
Ernster, L., Lindberg, O. & Löw, H. (1955). *Nature, Lond.*, **175**, 168.
Ernster, L., Ljunggren, M. & Lindberg, O. (1954). *Acta chem. scand.* **8**, 658.
Green, D. E., Beinert, H., Fuld, M., Goldmann, D., Paul, M. H. & Sarkar, N. K. (1953). *Exp. Cell Res.* **4**, 222.
Hatefi, Y. & Lester, R. L. (1958). *Biochim. biophys. Acta*, **27**, 83.
Hogeboom, G. H. (1955). In *Methods in Enzymology*, vol. 1, p. 16. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Holton, F. A., Hülsmann, W. C., Myers, D. K. & Slater, E. C. (1957). *Biochem. J.* **67**, 579.
Hülsmann, W. C. & Slater, E. C. (1957). *Nature, Lond.*, **180**, 372.
Jalling, O., Lindberg, O. & Ernster, L. (1955). *Acta chem. scand.* **9**, 198.
Korkes, S. (1952). In *Phosphorus Metabolism*, vol. 2, p. 618. Ed. by McElroy, W. D. & Glass, B. Baltimore: Johns Hopkins Press.
Korkes, S., del Campillo, A. & Ochoa, S. (1950). *J. biol. Chem.* **187**, 891.
Krebs, H. A., Ruffo, A., Johnson, M., Eggleston, L. V. & Hems, R. (1953). *Biochem. J.* **54**, 107.
Lewis, S. E. & Slater, E. C. (1954). *Biochem. J.* **58**, 207.
Maley, G. F. & Plaut, G. W. E. (1953). *J. biol. Chem.* **205**, 297.
Maley, G. F. & Plaut, G. W. E. (1954). *Biochim. biophys. Acta*, **14**, 443.
Massey, V. (1955). In *Methods in Enzymology*, vol. 1, p. 729. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Myers, D. K. & Slater, E. C. (1957). *Biochem. J.* **67**, 558.
Nielsen, S. O. & Lehninger, A. L. (1954). *J. Amer. chem. Soc.* **76**, 3860.
Nossal, P. M. (1951). *Biochem. J.* **49**, 407.
Nossal, P. M. (1952). *Biochem. J.* **50**, 349.
Packer, L. (1958). *Exp. Cell Res.* **15**, 551.
Slater, E. C. (1949). *Biochem. J.* **45**, 1.
Slater, E. C. (1953). *Biochem. J.* **53**, 157, 521.
Slater, E. C. (1954). *Nature, Lond.*, **174**, 1143.
Slater, E. C. (1955). *Biochem. J.* **59**, 392.
Slater, E. C. (1956). *Proc. 3rd. int. Congr. Biochem., Brussels*, p. 264.
Slater, E. C. & Bonner, W. D. (1952). *Biochem. J.* **52**, 185.
Slater, E. C. & Cleland, K. W. (1953). *Biochem. J.* **55**, 566.
Slater, E. C. & Holton, F. A. (1954). *Biochem. J.* **56**, 28.
van Dorp, A. & Slater, E. C. (1959). *Biochim. biophys. Acta*, **33**, 559.
Whittam, R., Bartley, W. & Weber, G. (1955). *Biochem. J.* **59**, 590.
Wu, C. Y. & Tsou, C. L. (1955). *Sci. Sinica*, **4**, 137.

Some Properties of the Malic Enzyme of Pigeon Liver

1. CONVERSION OF MALATE INTO PYRUVATE

By R. G. STICKLAND*
Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, University of Oxford

(*Received* 14 *April* 1959)

The malic enzyme, first described in pigeon liver by Ochoa, Mehler & Kornberg (1948), catalyses the reaction:

$$\text{Malate} + \text{TPN}^+ \xrightarrow{Mn^{2+}} \text{Pyruvate} + CO_2 + \text{TPNH}$$

where TPN^+ and TPNH stand for the oxidized and reduced forms of triphosphopyridine nucleotide. The enzyme is thought to play a role in the synthesis of glycogen from lactate and pyruvate and to be one of the factors controlling the supply of oxaloacetate in the liver (Krebs, 1954; Hiatt, Goldstein, Lareau & Horecker, 1958). Although the coenzyme requirements of the enzyme are firmly established relatively little is known about the kinetics and the effects of inhibitors. Rutter & Lardy (1958) have examined the effects of pH and cations on the conversion of malate into pyruvate.

* Present address: John Innes Horticultural Institution, Bayfordbury, Hertford, Herts.

A number of carboxylic acids, such as malonic acid, tartronic acid, mesoxalic acid, D- and *meso*-tartaric acid, oxalic acid, fluoromalic acid and fluorocitric acid, cause an accumulation of acetoacetate in respiring liver preparations (Edson, 1936; H. A. Krebs, unpublished work). Since ketogenesis may be related to the supply of oxaloacetate, the action of the above-mentioned acids might be due to the inhibition of either the 'oxaloacetate-synthesizing enzyme' (Utter & Kurahashi, 1955), which catalyses the reaction

$$\text{Phosphopyruvate} + CO_2 + (\text{GDP or IDP}) \xrightarrow{Mn^{2+}} \text{Oxaloacetate} + (\text{GTP or ITP})$$

(where GDP and GTP stand for guanosine di- and tri-phosphate and IDP and ITP for inosine di- and tri-phosphate), or malic enzyme, both of which lead to the formation of oxaloacetate from the products of glycolysis. The action of the ketogenic

acids on these two enzymes was therefore investigated.

The first paper of this series is concerned with a study of the kinetics of the decarboxylation of malate to pyruvate and of the effects of the ketogenic carboxylic acids on this reaction. Their effects on the synthesis of malate by malic enzyme and on the oxaloacetate-synthesizing enzyme are reported in subsequent papers (Stickland, 1959*a*, *b*).

EXPERIMENTAL

Preparation of enzyme. The enzyme was prepared from pigeon liver by the method of Ochoa (1955), modified in the first stages by Dr D. D. Davies (personal communication). The modifications consisted of the omission of the acetone precipitation; instead of this, some of the inactive cell fractions were removed by centrifuging. One of the samples of purified malic enzyme used was kindly given by Dr D. D. Davies.

Pigeon livers (150 g.) were homogenized in a stainless-steel homogenizer of the Potter–Elvehjem type in 2 vol. of cold 0·9% KCl. The homogenate was centrifuged at 650 *g* for 2 min.; the residue was suspended in 0·67 vol. of cold 0·9% KCl and recentrifuged (650 *g* for 2 min.). The combined supernatants (300 ml.) contained practically no material which could be sedimented at 2500 *g* in 60 min. and were used for the next step. Ammonium sulphate sufficient for full saturation (210 g.) was added slowly at 1° to the supernatants. The precipitate was centrifuged off, dissolved in 20 ml. of 0·02M-potassium phosphate buffer, pH 7·4, and dialysed for 24 hr. at 5° against the same buffer. The phosphate buffer was changed several times and stirred by a stream of air bubbles. A slight precipitate in the dialysed solution was centrifuged off and the supernatant used for the first ethanol fractionation as described by Ochoa. All centrifuging was done at 0°.

The total yield of enzyme was about 10% of that present initially. The specific activity of the final enzyme preparation was 6·5 times that of the supernatant from the dialysed solution. The assay was under the conditions shown in Fig. 1, which gave the maximum rate of reduction of oxidized triphosphopyridine nucleotide (TPN^+). The final protein concentration was 3·4 mg./ml., as measured by the biuret method with bovine serum albumin (The Armour Laboratories) as standard. The enzyme solution was diluted tenfold as required and 0·1 ml. used in the cuvette. The protein concentration in the reaction mixture was thus 0·011 mg./ml.

The enzyme preparation contained no measurable fumarase or malic dehydrogenase activity, but some lactic dehydrogenase activity. This impurity, however, did not interfere with the assay of the malic enzyme activity, since only initial rates were measured and the pyruvate concentration was negligible.

Special chemicals. Two preparations of TPN^+ were used (referred to as 'pure' and 'impure'). The pure preparation, obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A., was approx. 95% pure. The impure preparation, prepared according to the method of Kornberg & Horecker (1953), contained 50% of TPN^+ and 14% of diphosphopyridine nucleotide. The molar extinction coefficient of reduced triphosphopyridine nucleotide (TPNH) at 340 mμ was taken as 6220 (Kornberg & Horecker, 1953). Dr P. W. Kent kindly supplied 2-fluorocitrate as its triethyl ester; this was hydrolysed by heating with the theoretical amount of NaOH at 100° for 1 hr.

Measurement of enzyme activity. The reaction was followed by measuring the increase in absorption at 340 mμ, this being assumed to represent reduction of TPN^+. The reaction was carried out in 1 cm. silica cells in a Beckman spectrophotometer (model DU). The temperature of the cell was maintained at 30° by means of a thermostat cell-holder. Under the test conditions pyruvate had a molar extinction coefficient of 17 and malate of about 2. Change in absorption due to these compounds was ignored.

The reaction medium, which had a total volume of 3 ml., contained 0·1 ml. of enzyme, 0·3 μmole of TPN^+ and varying amounts of 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, malate, activators and inhibitors. Details are given with the individual experiments. The pH of the buffer given is that at 30°. L-Malate was used unless otherwise stated.

The reactants were added to the cell while it was in position in the cell-holder. All the reactants were at 30° before addition except for the enzyme and TPN^+, which were kept at 0°. After the addition of all the reactants except enzyme, 2 min. was allowed for temperature equilibration. During this equilibration period a zero-time reading was taken. The enzyme was added to start the reaction and the cell contents were stirred vigorously for 10–15 sec. The first reading was taken 30 sec. after the addition of the enzyme and subsequent readings were taken every 30 sec. for 4 min., or every 15 sec. for 2 min., depending on the speed of the reaction. No difference was found when the reaction was started by addition of TPN^+.

The initial rate was constant over at least five readings; all rates mentioned in the Results section are these initial rates.

Analysis of kinetics. The dissociation constants of enzyme–inhibitor complexes were obtained by the method of Dixon (1953). The terms $1/v$, $1/[S]$ and $[I]$ refer respectively to the reciprocal of the initial velocity, the reciprocal of the substrate concentration and to the inhibitor concentration.

RESULTS

Effect of malate concentration. The rate of TPN^+ reduction increased with the malate concentration until the latter reached about mM, and then fell. The shape of the curve varied with the Mn^{2+} ion concentration (Fig. 1). The peak of the curve was at a higher malate concentration when the $MnCl_2$ concentration was greater. Similar curves were obtained with Mg^{2+} ions as the cofactor, but the decrease in the rate at high malate concentration was not so great.

The inhibition of the reaction by a high concentration of malate was thought to be due to chelation of the bivalent cations by malate. To test this the Mn^{2+} ion concentration was increased, and as expected the rate at high malate concentration rose (Fig. 2). There was no inhibition by high malate concentration when the Mn^{2+} ion concentration was increased stoicheiometrically with the malate.

The shape of the curves also depended on pH (Fig. 3). At pH 7·7 the peak in the curve was higher and at a greater malate concentration. From Fig. 3 it appears that at higher malate concentrations (10 mM) the optimum pH is greater; this has been found to be so with the malic enzymes from cattle lens (van Heyningen & Pirie, 1953) and from pigeon liver (Rutter & Lardy, 1958).

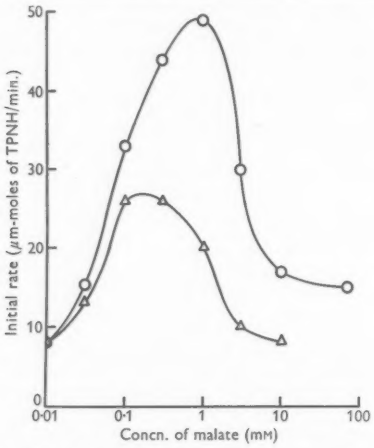


Fig. 1. Effect of malate concentration on malate decarboxylation. Cuvettes contained tris buffer, pH 7·2, (20 mM), TPN^+ (0·3 μmole), enzyme (0·1 ml.) and malate and $MnCl_2$ as indicated, in a total volume of 3 ml. △, 0·33 mM-$MnCl_2$; ○, 3·3 mM-$MnCl_2$.

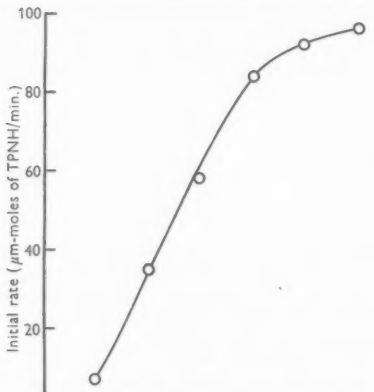


Fig. 2. Effect of increasing malate and $MnCl_2$ concentrations stoicheiometrically on malate decarboxylation. Cuvettes contained tris buffer, pH 7·4 (0·1 M), impure TPN^+ (0·3 μmole), enzyme (0·1 ml.) and malate and $MnCl_2$ as indicated, in a total volume of 3 ml.

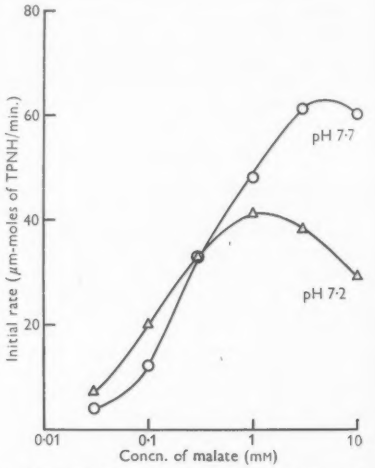


Fig. 3. Effect of pH on malate decarboxylation. Cuvettes contained tris buffer (0·02 M), $MgCl_2$ (3·3 mM), impure TPN^+ (0·3 μmole), enzyme (0·1 ml.) and malate as shown above, in a total volume of 3 ml.

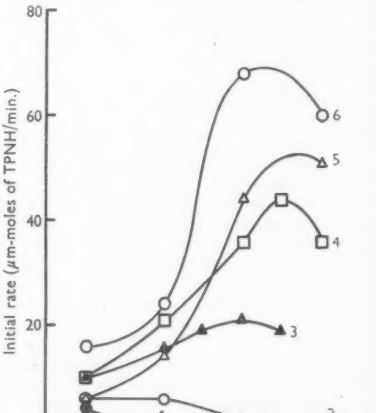


Fig. 4. Effect of metal salts on malate decarboxylation. Cuvettes contained tris buffer, pH 7·2 (0·02 M), malate (mM), impure TPN^+ (0·3 μmole), enzyme (0·1 ml.) and metal salts as indicated, in a total volume of 3 ml. Curve 1, $CuSO_4$; curve 2, $ZnSO_4$; curve 3, $NiSO_4$; curve 4, $CoCl_2$; curve 5, $MgCl_2$; curve 6, $MnCl_2$. The initial rate in the absence of added metal salt was 6 μm-moles of TPNH/min.

Table 1. *Effect of C_3-dicarboxylic acids on malate decarboxylation*

Each cuvette contained tris buffer, pH 7·4 (0·1 M), pure TPN^+ (0·3 μmole) and enzyme (0·1 ml.) in a total volume of 3 ml. Malate, inhibitor and metal salt concentrations were as given below. With mesoxalate the cation was Mg^{2+}, with the others Mn^{2+}.

Concn. of malate (mM)	Concn. of $MnCl_2$ (mM)	Inhibition (%) Malonate 20 mM	Malonate 5 mM	Malonate 2 mM	Tartronate 0·06 mM	Tartronate 0·03 mM
1·0	3·3	84	48	19	—	—
0·3	3·3	91	74	48	—	—
0·1	3·3	100	88	71	30	15
0·06	3·3	—	—	—	30	13
0·03	3·3	—	—	—	33	17
1·0	3·3	—	50	27	—	—
1·0	0·33	—	52	26	—	—

	Concn. of $MgCl_2$ (mM)	Mesoxalate mM	Mesoxalate 0·8 mM	Mesoxalate 0·5 mM	Mesoxalate 0·2 mM
1·0	3·3	60	50	40	15
0·6	3·3	—	63	52	23
0·3	3·3	—	74	62	43
1·0	0·03	69	—	—	—

Effect of cations. As already mentioned (Fig. 1), the initial rate of malate decarboxylation was greatly influenced by the Mn^{2+} ion concentration and further data are given in Fig. 4. Mg^{2+} and Co^{2+} ions have also been reported to activate malic enzyme (Salles & Ochoa, 1950; Conn, Vennesland & Kraemar, 1949). In the present experiments these ions, and also Ni^{2+} ions, were found to activate (Fig. 4). The optimum concentration was between 3 mM and 10 mM for all four ions. Concentrations over 10 mM caused some inhibition. Highest initial rates were obtained with Mn^{2+} ions. The effects of the other cations decreased in the order Mg^{2+}, Co^{2+} and Ni^{2+}. Zinc sulphate inhibited the enzyme at concentrations greater than 0·3 mM; this inhibition was not due to the SO_4^{2-} ion since $MnSO_4$ activated to the same extent as $MnCl_2$. Copper sulphate was an even stronger inhibitor, complete inhibition being caused by a concentration of 0·3 mM.

Zinc sulphate (0·3 mM) inhibited 70% in the presence of mM-Mn^{2+} ion, compared with 79% with 0·3 mM-Mn^{2+} ion.

Calcium chloride had little effect on the rate of reaction when added together with $MnCl_2$. With 0·33 mM-$MnCl_2$, 3·3 mM-$CaCl_2$ increased the initial rate from 52 to 56 μm-moles of TPNH/min. Calcium chloride (20 mM) inhibited approximately 90% when added alone.

The univalent ions, K^+, Na^+, Li^+ and NH_4^+, at a concentration of 0·02 M, had no effect on the reaction rate.

Effect of dicarboxylic acids. Malonate, tartronate and mesoxalate all inhibit the decarboxylation of malate (Table 1).

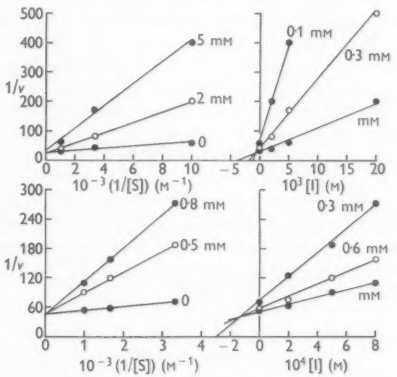


Fig. 5. Effect of malonate and mesoxalate on malate decarboxylation. Top graphs are for malonate, bottom graphs are for mesoxalate. Left-hand graphs are plots of $1/v$ against $1/[S]$; the figures on the graph indicate the inhibitor concentration. Right-hand graphs are plots of $1/v$ against [I]; the figures on the graphs indicate the substrate concentration. Tris buffer, TPN^+ and enzyme were as given in Table 1. With malonate, 3·3 mM-$MnCl_2$ was present, and with mesoxalate, 3·3 mM-$MgCl_2$. (v, Initial velocity as μmoles of TPNH formed in 1 min. [S] and [I] are substrate and inhibitor concentrations.)

Malonate is a mainly competitive inhibitor; in a $1/v$ against $1/[S]$ plot the lines cross almost on the ordinate (Fig. 5). Decreasing the malate concentration from mM to 0·1 mM increased the inhibition by 2 mM-malonate from 19 to 71% (Table 1). A tenfold decrease in $MnCl_2$ concentration did not alter the inhibition (27% at 3·3 mM and 26% at 0·3 mM-

$MnCl_2$), showing that malonate did not inhibit by chelating cations. Mesoxalate is also a competitive inhibitor (Fig. 5). Since mesoxalate forms an insoluble compound with $MnCl_2$, $MgCl_2$ was used as activator for the enzyme. Changing the malate concentration from mM to 0·3 mM at 0·5 mM-mesoxalate brought about an increase of inhibition from 40 to 62 %. With both malate and mesoxalate at mM, decrease in the $MgCl_2$ concentration from 3·3 mM to 0·033 mM only increased the inhibition from 60 to 69 %, which suggests that mesoxalate did not inhibit by binding cations.

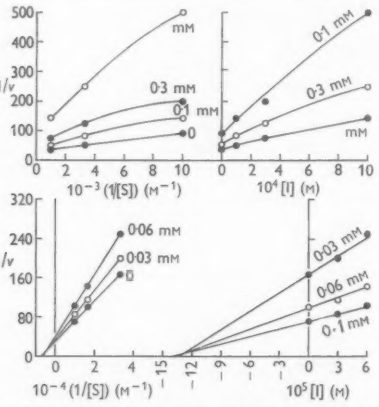


Fig. 6. Effect of tartronate on malate decarboxylation. Upper graphs show the effect at higher concentrations and the bottom graphs that at lower concentrations of tartronate. $MnCl_2$ concentration was 3·3 mM. Other details are as given in Fig. 5.

Table 2. *Values of K_i for various inhibitors of malic enzyme*

Values of K_i were obtained graphically by the method of Dixon (1953).

Inhibitor	K_i (mM)
Malonate	0·6
Tartronate	0·1
Mesoxalate	0·1
Succinate	20
L-Aspartate	10
*meso*Tartrate	2

The figures in Table 1 indicate that tartronate is a non-competitive inhibitor, the increase in inhibition by 0·06 mM-tartronate being only from 30 to 33 % when the malate was decreased from 0·1 mM to 0·03 mM. At high tartronate concentrations (mM), both $1/v$ against $1/[S]$ and $1/v$ against [I] plots were non-linear (Fig. 6). However, at concentrations of 0·1 mM and below these plots gave straight lines which crossed in the upper left quadrant of the graph, showing the inhibition must have some competitive element (Fig. 6). A part of the tartronate inhibition was due to the chelation of cations, as a tenfold decrease in $MnCl_2$ concentration (3·3 mM to 0·33 mM) increased the inhibition from 52 to 77 % in the presence of mM-malate and 0·3 mM-tartronate. The fact that tartronate appears to inhibit in two ways (by competition with malate and by chelation with Mn^{2+} ions) may explain the non-linearity of the $1/v$ against $1/[S]$ graph at high tartronate concentrations.

All three of the C_3-dicarboxylic acids gave K_i values of between 0·1 mM and 0·6 mM (see Table 2).

A number of C_4-dicarboxylic acids also inhibited malate decarboxylation. Succinate and L-aspartate inhibited to an equal extent (Table 3). With inhibitor concentrations of 20 mM, decrease in the malate concentration from 0·1 mM to 0·03 mM increased the inhibition from 46 to 52 % for succinate and from 52 to 57 % for aspartate. As for tartronate, the figures in Table 3 suggest that these inhibitors are largely non-competitive, but for succinate a $1/v$ against $1/[S]$ plot (in which the lines crossed in the upper left quadrant) showed the inhibition to be partially competitive (Fig. 7). The graph for aspartate was non-linear (Fig. 7). At the same inhibitor concentration as above (20 mM) and 0·1 mM-malate, decreasing the $MnCl_2$ concentration from 3·3 mM to 0·33 mM changed the inhibition due to succinate from 46 to 56 % and that due to aspartate from 52 to 50 %. The K_i values for these two inhibitors were about 10 mM (Table 2).

DL-Malate gave the same initial rate as half its concentration of L-malate. D-Malate thus had no effect when present in a concentration equal to that of L-malate.

*meso*Tartrate had an inhibitory power approximately midway between those of succinate and the

Table 3. *Effect of succinate and aspartate on malate decarboxylation*

Cuvette contents were as in Table 1, except for $MnCl_2$, malate and inhibitor, which were as given below.

Concn. of malate (mM)	Concn. of $MnCl_2$ (mM)	Inhibition (%)					
		Succinate			L-Aspartate		
		30 mM	20 mM	10 mM	20 mM	10 mM	4 mM
0·1	3·3	54	46	31	52	32	12
0·06	3·3	56	44	28	52	36	14
0·03	3·3	57	52	24	57	48	9
0·1	0·33	—	56	—	50	—	—

C_3-acids (Table 4); its value for K_i was about mM. The inhibition by *meso*tartrate was competitively reversed by malate (Fig. 8 and Table 4). With mM-*meso*tartrate there was 33% inhibition with 0·02 mM-malate but only 4% inhibition with 0·1 mM-malate. To some extent its effect was due to chelation of cations: the 4% inhibition quoted above was increased to 28% if the Mn^{2+} ion concentration was decreased 50-fold. This increase was not so great (48–59%) with 3 mM-*meso*tartrate.

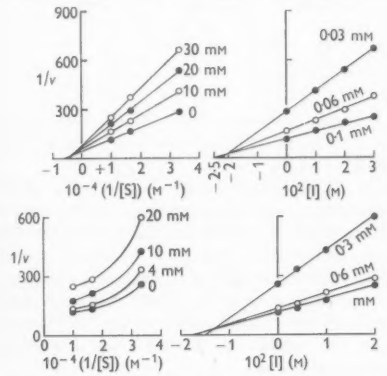


Fig. 7. Effect of succinate and L-aspartate on malate decarboxylation. Upper graphs refer to succinate and the lower ones to L-asparate. Other details are as given in Fig. 5 (3·3 mM-$MnCl_2$ was present).

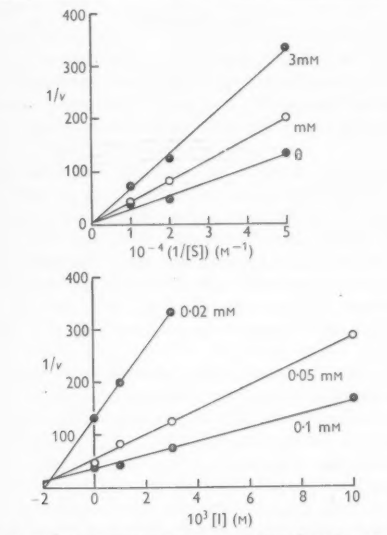


Fig. 8. Effect of *meso*tartrate on malate decarboxylation. Plots of $1/v$ against 1/[S] (on which the figures indicate the inhibitor concentration) and of $1/v$ against [I] (the figures giving the substrate concentration) for *meso*tartrate. Tris buffer, TPN^+ and enzyme were as given in Table 1. The $MnCl_2$ concentration was 3·3 mM. v, Initial velocity (μmoles of TPNH formed in 3 min.).

Inhibition by oxaloacetate was much more powerful. Marked inhibition was obtained with oxaloacetate concentrations less than the malate concentration (Table 5). An oxaloacetate concentration of 0·167 mM inhibited 66% with mM-malate and 91% with 0·1 mM-malate. Decreasing the $MnCl_2$ concentration to one-tenth (to 0·33 mM) had no effect on the inhibition (see Table 5). The inhibition due to oxaloacetate was thus competitive and did not involve the chelation of cations. In these experiments, Mn^{2+} ions were present together with the oxaloacetate, and it is known that oxaloacetate can be decarboxylated non-enzymically in the presence of Mn^{2+} ions (Krebs, 1942). The extinction at 290 mμ was used as a measure of the oxaloacetate concentration in the solution since, at this wavelength, oxaloacetate absorbs, but TPNH has very little absorption. During the time of the temperature equilibration and the reaction, the decrease in the oxaloacetate concentration was always less than 10%.

As shown in Table 6, D-tartrate inhibited to about the same extent as *meso*tartrate. With 0·1 mM-malate, D-tartrate concentrations of 2 mM inhibited 56% and of 0·6 mM, 25%. Inhibition by fumarate and maleate was relatively weak. With the inhibitor concentration (10 mM) 100 times the malate concentration (0·1 mM), fumarate inhibited

Table 4. *Effect of* meso*tartrate on malate decarboxylation*

Cuvette contents were as in Table 1, except for $MnCl_2$, malate and *meso*tartrate, which were as given below.

Concn. of malate (mM)	Concn. of $MnCl_2$ (mM)	Inhibition (%) by *meso*tartrate		
		10 mM	3 mM	mM
0·1	3·3	77	48	4
0·05	3·3	83	61	41
0·02	3·3	100	60	33
0·1	0·066	—	59	28

Table 5. *Effect of oxaloacetate on malate decarboxylation*

Cuvette contents were as in Table 1, except for $MnCl_2$, malate and oxaloacetate, which were as given below.

Concn. of malate (mM)	Concn. of $MnCl_2$ (mM)	Inhibition (%) by oxaloacetate	
		0·167 mM	0·067 mM
1	3·3	66	49
0·1	3·3	91	79
1	0·33	64	—

Table 6. *Effect of other C_4-dicarboxylic acids on malate decarboxylation*

Cuvette contents were as in Table 1, except for $MnCl_2$, malate and inhibitors, which were as given below.

Concn. of malate (mM)	Concn. of $MnCl_2$ (mM)	Inhibition (%)				
		D-Tartrate			Fumarate (10 mM)	Maleate (10 mM)
		10 mM	2 mM	0·6 mM		
1	3·3	65	24	—	8	8
0·1	3·3	—	56	25	31	21
0·1	0·033	—	—	29	53	28

Table 7. *Effect of oxalate on malate decarboxylation*

Cuvettes contained tris buffer, pH 7·2 (0·02M), $MnCl_2$ (0·02M), impure TPN^+ (0·3 μmole) and enzyme (0·1 ml.) in a total volume of 3 ml. Malate and oxalate were as given below.

Concn. of malate (mM)	Inhibition (%) by oxalate		
	3 mM	2 mM	0·75 mM
10	72	63	—
1	77	—	56

Table 8. *Effect of citrate on malate decarboxylation*

$MnCl_2$, malate and citrate were as given below; other contents were as given in Table 1.

Concn. of malate (mM)	Concn. of $MnCl_2$ (mM)	Inhibition (%) by citrate	
		20 mM	10 mM
1	3·3	28	25
0·1	3·3	23	6
0·1	0·033	95	—

31 % and maleate 21 %. These two figures were increased to 53 and 28 % respectively when the $MnCl_2$ concentration was reduced from 3·3 mM to 0·033 mM.

Oxalate proved a strong inhibitor of the enzyme (Table 7). At 3 mM-oxalate, there was 72 % inhibition in the presence of 10 mM-malate and 77 % inhibition in the presence of mM-malate.

Effect of other carboxylic acids on malate decarboxylation. Citrate (20 mM) inhibited 23 % at a $MnCl_2$ concentration of 3·3 mM and with 0·1 mM-malate (Table 8). At a $MnCl_2$ concentration only 1 % of this, there was almost complete inhibition (95 %), indicating that a large part of the inhibition was due to the binding of Mn^{2+} ions.

Citrate required concentrations in excess of 10 mM for appreciable inhibition. Fluorocitrate, however, inhibited to the same extent at concentrations of mM (Table 9). Thus with mM-fluorocitrate there was 35 % inhibition with 10 mM-malate and 76 % inhibition with mM-malate (the $MnCl_2$ concentration was 0·33 mM). The inhibition was competitive, but, as with citrate, some of the inhibition also appeared to be due to chelation with Mn^{2+} ions. With both fluorocitrate and malate at a concentration of mM, decrease of the $MnCl_2$ concentration from 17 mM to 0·33 mM increased the inhibition from 34 to 76 %.

Four C_3-monocarboxylic acids were studied: propionate, D- and L-lactate and pyruvate. At a concentration of 10 mM in the presence of mM-malate, propionate did not inhibit, D- and L-lactate caused identical inhibition (23 %) and pyruvate inhibited 16 % (Table 10). A tenfold increase in either the malate or the $MnCl_2$ concentration had almost no effect on the inhibition by any of these acids.

DL-β-Hydroxybutyrate (10 mM) caused no inhibition and DL-α-hydroxybutyrate (10 mM) inhibited 19 % under the conditions given above for lactate inhibition (see Table 10).

DISCUSSION

About 50 % inhibition of the initial rate of conversion of malate into pyruvate was obtained with oxaloacetate at 0·01 mM, malonate, tartronate and mesoxalate at 0·3 mM, oxalate and fluorocitrate at about mM, *meso*tartrate at 3 mM, D-tartrate and fumarate at 10 mM and succinate and L-aspartate at 20 mM. Maleate, citrate, D- and L-lactate, pyruvate and DL-α-hydroxybutyrate inhibited about 20 % at a concentration of 10 mM. Under the same conditions propionate and DL-β-hydroxybutyrate did not inhibit. These data apply to an L-malate concentration of 0·1 mM, except for oxalate and fluorocitrate, which refer to mM-L-malate.

The inhibitions of the malic enzyme appear to consist of two different elements: the chelation of the bivalent cations (Mn^{2+} and Mg^{2+}), which act at cofactors for the enzyme, and a true inhibition of the enzyme. That chelation of ions plays a role is suggested by the fact that the inhibition can be partly overcome by the addition of cations. This applies especially to the inhibition by citrate, by high concentrations of malate and to a less extent to the other inhibitory substances, as judged by the reduction of the inhibition on addition of Mn^{2+} or Mg^{2+} ions. The analysis of the inhibitions by the methods indicated by Dixon (1953) shows that the inhibitions were partly competitive and partly

Table 9. *Effect of fluorocitrate on malate decarboxylation*

Cuvettes contained tris buffer (pH 7·2, 0·02 M in Expt. 1; pH 7·4, 0·1 M in Expt. 2), pure TPN^+ (0·3 μmole) and enzyme (0·1 ml.) in a total volume of 3 ml. Malate, $MnCl_2$ and fluorocitrate were as given below.

Expt. no.	Concn. of malate (mM)	Concn. of $MnCl_2$ (mM)	Inhibition (%) by fluorocitrate				
			10 mM	4 mM	mM	0·1 mM	0·01 mM
1	10	17	—	—	27	—	—
	10	0·33	—	—	35	—	—
	1	17	—	—	34	—	—
	1	0·33	—	—	76	—	—
2	1	3·3	84	—	36	14	2
	1	0·33	—	84	59	14	0

Table 10. *Effect of monocarboxylic acids on malate decarboxylation*

Cuvette contents were as given in Table 1, except for $MnCl_2$ and malate, which were as given below. Concentration of the monocarboxylic acids was 10 mM.

Concn. of malate (mM)	Concn. of $MnCl_2$ (mM)	Inhibition (%)					
		Propionate	D-Lactate	L-Lactate	Pyruvate	DL-α-Hydroxy-butyrate	DL-β-Hydroxy-butyrate
1	3·3	0	23	23	16	19	0
0·1	3·3	0	22	22	18	21	0
0·1	0·33	0	23	23	19	—	—
0·1	0·033	—	—	—	—	28	0

non-competitive, which could be explained if the two different elements were responsible for the inhibition. The competitive element is not unexpected since all the effective inhibitors resemble malate structurally in that they possess either two carboxyl groups, or one carboxyl plus an α-hydroxyl or carbonyl group. These substances presumably compete with the malate for the binding sites of the enzyme.

Gal (1958) found very little inhibition of the malic enzyme by fluorocitrate, but the highest concentration tried by him was 0·066 mM. He found a 60% inhibition by 0·7 mM-fluoromalate. The only other carboxylic acids which have previously been shown to inhibit malate decarboxylation are malonate and fumarate (van Heyningen & Pirie, 1953). The same authors showed that impure sodium ethyl oxaloacetate was inhibitory.

The exact mechanism of the malic enzyme reaction is still unknown, but an oxaloacetate–enzyme complex is the most probable intermediate (see Rutter & Lardy, 1958). In this connexion it is perhaps significant that oxaloacetate was by far the most powerful inhibitor of malic enzyme found. The decarboxylation of oxaloacetate by malic enzyme is known to be inhibited by malonate, D- and L-malate, succinate and fumarate (Salles & Ochoa, 1950), but whether the same site on the enzyme is involved in this reaction is not known.

The activation of the enzyme by cations was greatest by Mn^{2+} ions, Mg^{2+}, Co^{2+} and Ni^{2+} ions being less effective; Cu^{2+} and Zn^{2+} were inhibitory. In the experiments of Rutter & Lardy (1958) the most active ion was Co^{2+}. They obtained slight inactivation by Zn^{2+} and Cu^{2+} ions. These differences might be due to the greater purity of the enzymes used by Rutter & Lardy, or to the presence of cysteine in their assay medium.

SUMMARY

1. Some aspects of the kinetics, and of the inhibition by carboxylic acids, of the conversion of L-malate into pyruvate have been investigated.

2. Mn^{2+} ions greatly increased the rate of malate decarboxylation. Mg^{2+}, Co^{2+} and Ni^{2+} ions also activated the enzyme, but the maximum rate obtained was less than with Mn^{2+} ions. Cu^{2+}, Zn^{2+} and Ca^{2+} ions inhibited the enzyme.

3. The decarboxylation of malate was inhibited by high malate concentrations. Since the inhibition was reversed by increased Mn^{2+} ion concentrations, it was probably due to chelation of Mn^{2+} ions by the malate.

4. All the dicarboxylic acids tested inhibited the reaction. The inhibitory power decreased in the following order: oxaloacetic acid (which inhibited at concentrations less than that of the substrate), tartronic acid, mesoxalic acid, malonic acid, oxalic acid, *meso*tartaric acid, D-tartaric acid, L-aspartic acid, succinic acid, fumaric acid and maleic acid. For the last four acids concentrations 100 times that of the substrate were required for appreciable inhibition.

5. D- and L-Lactate, pyruvate and DL-α-hydroxybutyrate inhibited slightly at high concentrations. Propionate and DL-β-hydroxybutyrate were not inhibitory at the same concentrations.

6. Citrate and fluorocitrate also inhibited malate decarboxylation. For equal inhibition, the concentration of citrate required was over ten times that of the fluorocitrate.

7. The inhibition by di- and tri-carboxylic acids was partly reversed by addition of Mn^{2+} (or Mg^{2+}) ions and partly by raising the substrate concentration. The data are in accordance with the view that the inhibitions are due partly to chelation of the bivalent cations required as cofactors and partly to competition with the malate for the enzyme-binding site.

The author wishes to thank Professor Sir Hans Krebs, F.R.S., and the members of the Medical Research Council Unit for their advice and interest concerning the work reported in these three papers. The work was carried out during the tenure of an Agricultural Research Council Research Studentship, and this support is gratefully acknowledged.

REFERENCES

Conn, E., Vennesland, B. & Kraemar, L. M. (1949). *Arch. Biochem.* **23**, 179.
Dixon, M. (1953). *Biochem. J.* **55**, 170.
Edson, N. L. (1936). *Biochem. J.* **30**, 1855.
Gal, E. M. (1958). *Arch. Biochem. Biophys.* **73**, 279.
Hiatt, H. H., Goldstein, M., Larcau, J. & Horecker, B. L. (1958). *J. biol. Chem.* **231**, 303.
Kornberg, A. & Horecker, B. L. (1953). *Biochem. Prep.* **3**, 24.
Krebs, H. A. (1942). *Biochem. J.* **36**, 303.
Krebs, H. A. (1954). *Johns Hopk. Hosp. Bull.* **95**, 19.
Ochoa, S. (1955). In *Methods in Enzymology*, vol. 1, p. 739. New York: Academic Press Inc.
Ochoa, S., Mehler, A. H. & Kornberg, A. (1948). *J. biol. Chem.* **174**, 979.
Rutter, W. J. & Lardy, H. A. (1958). *J. biol. Chem.* **233**, 374.
Salles, J. B. V. & Ochoa, S. (1950). *J. biol. Chem.* **187**, 849.
Stickland, R. G. (1959*a*). *Biochem. J.* **73**, 654.
Stickland R. G. (1959*b*). *Biochem. J.* **73**, 660.
Utter, M. F. & Kurahashi, K. (1955). In *Methods in Enzymology*, vol. 1, p. 758. New York: Academic Press Inc.
van Heyningen, R. & Pirie, A. (1953). *Biochem. J.* **53**, 436.

Some Properties of the Malic Enzyme of Pigeon Liver

2. SYNTHESIS OF MALATE FROM PYRUVATE

By R. G. STICKLAND*
Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, University of Oxford

(*Received* 14 *April* 1959)

The kinetics of the decarboxylation of malate by the malic enzyme of pigeon liver and of the inhibition of the reaction by carboxylic acids was the subject of the preceding paper (Stickland, 1959). In this paper are reported the results of a similar study on the synthesis of malate by the reaction:

$$\text{Pyruvate} + CO_2 + \text{TPNH} \xrightarrow[\text{Malic enzyme}]{Mn^{2+} \text{ or } Mg^{2+}} \text{Malate} + \text{TPN}^+,$$

where TPNH and TPN^+ stand for reduced and oxidized triphosphopyridine nucleotide respectively.

EXPERIMENTAL

Enzyme preparation. The enzyme preparation was the same as that used previously (Stickland, 1959). The preparation contained considerable lactic dehydrogenase activity. The rates observed were therefore corrected for the rate of oxidation of reduced triphosphopyridine nucleotide (TPNH) by pyruvate in the absence of added bicarbonate. Under these conditions the malic enzyme activity was not completely abolished since the solutions were not completely free from CO_2. Thus the corrections represented the maximum value for the activity of lactic dehydrogenase.

Special chemicals. Oxidized triphosphopyridine nucleotide (TPN^+) and TPNH (enzymically reduced) were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. Another sample of TPNH was prepared from TPN^+ by reduction with dithionite (Beisenherz *et al.* 1953). As indicated by absorption at 340 mμ the commercial preparation contained about 73% of TPNH by weight and the sample prepared chemically contained TPNH equal to about 80% of the TPN^+ added. The molar extinction coefficient of TPNH was taken as 6220 (Kornberg & Horecker, 1953).

The fluorocitrate preparation used was described previously (Stickland, 1959).

Measurement of enzyme activity. The reaction was followed as a decrease in absorption at 340 mμ (this being assumed to represent oxidation of TPNH) and was carried out in the apparatus used for the study of malate decarboxylation

* Present address: John Innes Horticultural Institution, Bayfordbury, Hertford, Herts.

(Stickland, 1959). Under the test conditions pyruvate had a molar extinction coefficient at 340 mμ of 17. As a result pyruvate concentrations greater than 50 mM could not be used, as the absorption was too high. Changes in absorption due to change in the pyruvate concentration were ignored.

The reaction medium, which had a total volume of 3 ml., contained 0·1 ml. of enzyme, 0·2 ml. of TPNH (0·15 μmole), 0·1 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, pH 7·4 at 30°, and varying amounts of pyruvate, bicarbonate, activators and inhibitors. Details are given with the individual experiments.

The reactants were added while the cell was in the cell-holder. All reactants were at 30° before addition, except for the enzyme and TPNH, which were kept at 0°. A CO_2+N_2 gas mixture was bubbled through the cell during the whole of the equilibration period and during the reaction. The bicarbonate concentration in the cell and the percentage of CO_2 in the gas phase were adjusted to give pH 7·4. The required concentrations were read from the graph given by Umbreit (1949). After temperature equilibration for at least 2 min. the TPNH was added and readings were taken to observe any non-enzymic rate of TPNH removal. Enzyme was added 3 min. after the addition of TPNH and readings were taken every 30 sec. for 4 or 5 min. The initial rate was constant over at least five readings; all rates mentioned in the Results section are these initial rates.

Analysis of kinetics. Apparent Michaelis constants obtained under one set of conditions only were determined graphically by the method of Lineweaver & Burk (1934). The type of inhibition was established by the graphical method of Dixon (1953).

RESULTS

Non-enzymic removal of TPNH. Preliminary studies on the oxidation of TPNH by pyruvate and bicarbonate–CO_2 mixtures showed that chemically reduced TPNH disappeared in the absence of enzyme, particularly in the presence of high concentrations of $MnCl_2$ or bicarbonate. This removal was catalysed readily by Mn^{2+}, Co^{2+}, Ni^{2+} and Cu^{2+} ions, but only to a small extent by Mg^{2+}, Zn^{2+} and Ca^{2+} ions (Table 1). The rate of removal in the presence of Mn^{2+}, Co^{2+} and Ni^{2+} ions was increased by bicarbonate (from 3·5 to 40 μm-moles of TPNH/3 min. for 3·3 mM-Mn^{2+} ions). Cu^{2+} ions were much more effective: in the absence of bicarbonate 0·33 mM-Cu^{2+} ions gave an initial rate of 43 μm-moles/3 min., whereas ten times this concentration of Mn^{2+} ions gave a rate of only 2 μm-moles/3 min. (Table 1, Expt. 3). However, bicarbonate decreased the rate in the presence of 0·33 mM-Cu^{2+} ions from 43 to 36 μm-moles of TPNH/3 min. In the presence of bicarbonate the rates with 0·33 mM-Cu^{2+} ions and 3·3 mM-Mn^{2+} ions were not very different, being 36 and 25 μm-moles/3 min. respectively.

Rackis & Kalnitsky (1957) found a non-enzymic oxidation of α-oxoglutarate by Mn^{2+} and Co^{2+} ions, which was stimulated by amino acids, particularly threonine. Threonine increased the rate of non-enzymic removal of TPNH in the presence of

Table 1. *Non-enzymic oxidation of reduced triphosphopyridine nucleotide*

Cuvettes contained tris buffer, pH 7·4 (0·1 M), TPNH (0·15 μmole) and metal salts, bicarbonate and threonine as indicated. In Expts. 1–3 chemically reduced TPNH was used; in Expt. 4 enzymically reduced TPNH was used. $NaHCO_3$ was 0·067 M; when $NaHCO_3$ was added CO_2+N_2 (20:80) was bubbled through cuvettes (pH 7·4). Threonine was 3·3 mM.

Expt. no.	Additions	Initial rate of TPNH oxidation (μm-moles/3 min.) No $NaHCO_3$ or threonine	+ $NaHCO_3$	+ Threonine
1	None	0	1	—
	$MnCl_2$ (3·3 mM)	3·5	40	—
	$CaCl_2$ (3·3 mM)	1	3·5	—
	$MgCl_2$ (3·3 mM)	1	1·5	—
	$ZnSO_4$ (3·3 mM)	0	2·5	—
2	$MnCl_2$ (3·3 mM)	4	33	—
	$CoCl_2$ (0·33 mM)	7	16	—
	$NiSO_4$ (0·33 mM)	6	23	—
3	None	0	2·5	2
	$MnCl_2$ (3·3 mM)	2	25	10
	$CuSO_4$ (0·33 mM)	43	36	6
4	None	0	0	0
	$MnCl_2$ (3·3 mM)	0	0	0
	$CuSO_4$ (0·33 mM)	1	0	—
	'Oxidized dithioniate' (0·4 mM)	0	2	—
	'Oxidized dithioniate' (0·4 mM) + $MnCl_2$ (3·3 mM)	2	19	—
	Na_2SO_3 (0·5 mM)	1	2	—
	Na_2SO_3 (0·5 mM) + $MnCl_2$ (3·3 mM)	6	60	—
	Na_2SO_3 (0·5 mM) + $CuSO_4$ (0·33 mM)	64	11	—

Mn^{2+} ions from 2 to 10 μm-moles/3 min.; that in the presence of Cu^{2+} ions was decreased from 43 to 6 μm-moles/3 min.

Mn^{2+} and Cu^{2+} ions caused almost no removal of enzymically reduced TPNH (Table 1, Expt. 4). During the chemical reduction of TPN^+, the dithionite used is oxidized mainly to sulphite. Assuming all the dithionite was oxidized to sulphite, the final concentration of sulphite in the cell was approximately 0·4 mM. The removal of enzymically reduced TPNH plus either 0·4 mM-'oxidized dithionite' (dithionite treated in the same manner as in TPN^+ reduction, but in the absence of TPN^+) or 0·5 mM-sulphite had the same characteristics as that of chemically reduced TPNH. The rate with Mn^{2+} ions was increased by bicarbonate (6–60 μm-moles/3 min.) and the rate with Cu^{2+} ions was decreased by bicarbonate (64–11 μm-moles/3 min.) (Table 1, Expt. 4). Thus the above-mentioned effects of the cations on chemically reduced TPNH were presumably dependent on the presence of sulphite. In all subsequent experiments enzymically reduced TPNH was used.

Effect of substrate concentration. The slow rate of TPNH oxidation in the absence of added bicarbonate was rapidly raised when the bicarbonate–CO_2 concentration was increased, a maximum being reached at about 0·15M-$NaHCO_3$ (45% of CO_2) (Fig. 1). Higher bicarbonate concentrations caused some inhibition.

Fig. 1. Effect of $NaHCO_3$ and CO_2 on malate synthesis. Cells contained tris buffer, pH 7·4 (0·1M), $MnCl_2$ (0·33 mM), TPNH (0·15 μmole) and enzyme (0·1 ml.) in a total volume of 3 ml., CO_2 was in the gas phase as shown above. Concentration of $NaHCO_3$ was varied to keep the pH at 7·4. ●, No pyruvate; △, 10 mM-pyruvate; ○, 50 mM-pyruvate. There was no precipitation of Mn^{2+} ions at the higher bicarbonate concentration.

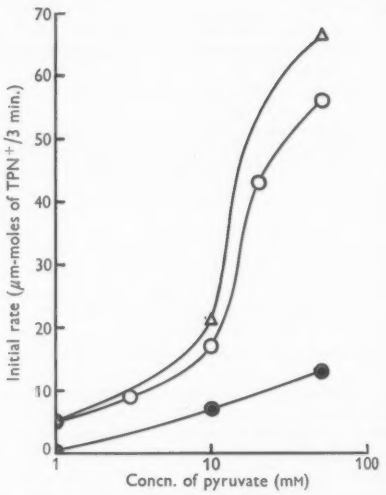


Fig. 2. Effect of pyruvate concentration on malate synthesis. ●, No CO_2 or $NaHCO_3$; ○, 20% of CO_2 and 66 mM-$NaHCO_3$; △, 60% of CO_2 and 0·198M-$NaHCO_3$. Other conditions were as given in Fig. 1. In the absence of pyruvate the initial rate was approx. 0·5 μm-mole of TPN^+/3 min.

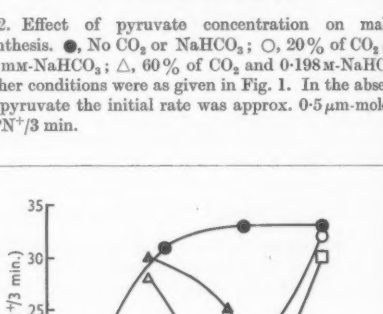


Fig. 3. Effect of metal salts on malate synthesis. Cells contained tris buffer, pH 7·4 (0·1M), pyruvate (10 mM), $NaHCO_3$ (66 mM), TPNH (0·15 μmole), enzyme (0·1 ml.) and metal salts as shown, in a total volume of 3 ml. ●, $MnCl_2$; ○, $MgCl_2$; □, $CoCl_2$; ▲, $CuSO_4$; △, $ZnSO_4$. $CuSO_4$ and $ZnSO_4$ curves were obtained in the presence of 0·033 mM-$MnCl_2$. The initial rate in the absence of added metal salt was approx. 10 μm-moles of TPN^+/3 min.

Table 2. *Effect of C_3-dicarboxylic acids on malate synthesis*

Cuvettes contained tris buffer, pH 7·4 (0·1 M), $NaHCO_3$ (0·2 M), TPNH (0·15 μmole), enzyme (0·1 ml.) and pyruvate, metal salts and inhibitors as given below, in a total volume of 3 ml. Gas phase, $CO_2 + N_2$ (60:40). With mesoxalate Mg^{2+} ions were used as activator; with the other inhibitors Mn^{2+} ions were used.

Concn. of pyruvate (mM)	Concn. of $MnCl_2$ (mM)	Inhibition (%) Malonate 10 mM	Malonate mM	Tartronate 0·1 mM	Tartronate 0·01 mM
50	0·33	46	10	70	24
10	0·33	70	37	74	32
1	0·33	62	43	—	—
50	0·0033	—	—	43	3

	Concn. of $MgCl_2$ (mM)	Mesoxalate 20 mM	5 mM	mM
50	3·3	65	45	30
10	3·3	43	36	12
50	0·033	—	50	21

In the absence of pyruvate there was no TPNH oxidation (Fig. 2). Values for apparent K_m of pyruvate and CO_2 were 2×10^{-2} M and 2×10^{-3} M respectively. The figure for CO_2 was calculated from the percentage of CO_2 in the gas phase required to give half the maximum rate.

Requirement for bivalent cations. The presence of bivalent cations was necessary for the maximum rate of malate synthesis. On increasing the $MnCl_2$ concentration a maximum rate was reached at approximately 0·1 mM (Fig. 3). Higher $MnCl_2$ concentrations (up to 3·3 mM) did not cause any inhibition. Mg^{2+} and Co^{2+} ions also activated the enzyme. At 0·033 mM they had little effect, but on increasing their concentrations the rate of reaction rose. A maximum rate was not reached by a concentration of 3·3 mM. Nickel sulphate activated the enzyme to the same extent as $CoCl_2$ at a concentration of 0·33 mM.

Copper sulphate and $ZnSO_4$ both inhibited when added together with 0·033 mM-$MnCl_2$. At a concentration of 2 mM, $CuSO_4$ and $ZnSO_4$ decreased the initial rate from 31 to 15 and 4 μm-moles/3 min. respectively.

Effect of carboxylic acids. Malonate, tartronate and mesoxalate inhibited the synthesis of malate. The degree of inhibition depended in some cases on the substrate concentration. Thus on decreasing the pyruvate concentration from 50 mM to 10 mM, the inhibition by mM-malonate was increased from 10 to 37 %, that by 0·1 mM-tartronate remained about the same (70–74 %) and that by 20 mM-mesoxalate was decreased from 65 to 43 % (Table 2). These figures suggest that the inhibition by malonate was competitive and that that by tartronate had a large non-competitive element. The tartronate inhibition was decreased by lowering the $MnCl_2$ concentration: with 50 mM-pyruvate and 0·1 mM-tartronate, the inhibition was 70 % at 0·33 mM-$MnCl_2$ and 43 % at 3·3 μM-$MnCl_2$. Since the mesoxalate inhibition decreases with decreasing substrate concentration, it appears that the inhibition is uncompetitive (see Ebersole, Guttentag & Wilson, 1944). However, in all cases plots of the reciprocal of the velocity ($1/v$) against inhibitor concentration and of $1/v$ against the reciprocal of the substrate concentration were non-linear (see Fig. 4), showing that first-order kinetic theory does not apply.

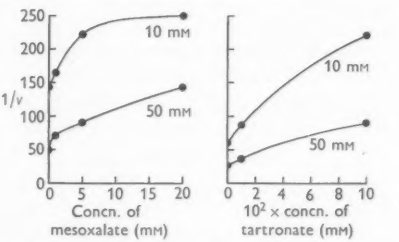


Fig. 4. Effect of tartronate and mesoxalate. Plots of reciprocal of velocity against mesoxalate and tartronate concentrations. The figures on the graphs indicate the pyruvate concentration. v, Initial velocity as μmoles of TPN^+ formed/3 min.

Three C_4-dicarboxylic acids (succinic acid, *meso*-tartaric acid and oxaloacetic acid) were competitive in as much as the inhibition depended on the substrate concentration. Decrease in the pyruvate concentration from 50 to 10 mM resulted in increased inhibition by 20 mM-succinate (31–47 %), 10 mM-*meso*tartrate (14–24 %) and 10 mM-oxaloacetate (47–62 %) (Table 3).

Inhibition by D- and L-lactate and DL-α-hydroxybutyrate also depended on the pyruvate concentration (Table 4). Thus with two pyruvate

Table 3. *Effect of C_4-dicarboxylic acids on malate synthesis*

Cuvette contents were as given in Table 2; $MnCl_2$ concentration was 0·33 mM. Pyruvate and inhibitors were as given below.

Concn. of pyruvate (mM)	Inhibition (%)					
	Succinate		*meso*Tartrate		Oxaloacetate	
	50 mM	10 mM	10 mM	2 mM	10 mM	mM
50	31	0	14	—	47	6
10	47	11	24	2	62	26

Table 4. *Effect of monocarboxylic acids on malate synthesis*

Cuvette contents were as given in Table 3.

Conc. of pyruvate (mM)	Inhibition (%)						
	D-Lactate		L-Lactate (20 mM)	Propionate (20 mM)	DL-α-Hydroxybutyrate		DL-β-Hydroxybutyrate (20 mM)
	20 mM	5 mM			20 mM	5 mM	
50	23	9	23	0	26	6	—
10	50	13	47	0	39	17	2

Table 5. *Effect of citrate and fluorocitrate on malate synthesis*

Cuvette contents were as given in Table 2, except pyruvate, $MnCl_2$ and inhibitors, which were as given below.

Concn. of pyruvate (mM)	Concn. of $MnCl_2$ (mM)	Inhibition (%)		
		Fluorocitrate		Citrate (20 mM)
		0·167 mM	0·0167 mM	
50	0·33	39	11	25
10	0·33	50	25	13
50	0·033	28	—	44

Table 6. *Comparison of the inhibitory effects of di- and tri-carboxylic acids on the decarboxylation and synthesis of malate*

Values for malate decarboxylation are from Stickland (1959) and were obtained in the presence of 0·1 mM-malate. For malate synthesis, 10 mM-pyruvate was used. These differing substrate concentrations were necessary in order to obtain measurable rates of reaction.

Inhibitor	Concn. of inhibitor (mM) required for 50% inhibition of the initial rate of	
	Malate decarboxylation	Malate synthesis
Oxaloacetate	0·01	10–1
Malonate	0·3	1
Tartronate	0·3	0·10–0·01
Mesoxalate	0·3	20
Oxalate	1·0	—
Fluorocitrate	1·0	0·1
*meso*Tartrate	3·0	>10
D-Tartrate	10·0	—
Fumarate	10·0	—
Succinate	20·0	50
L-Aspartate	20·0	—

concentrations of 50 mM and 10 mM, 20 mM-D-lactate inhibited 23 and 50%, 20 mM-L-lactate 23 and 47% and 20 mM-DL-α-hydroxybutyrate 26 and 39% respectively. Propionate (20 mM) did not inhibit. At the lower of these pyruvate concentrations there was virtually no inhibition (2%) by 20 mM-β-hydroxybutrate.

The inhibition by fluorocitrate and citrate depended on the substrate concentration, but that by 0·167 mM-fluorocitrate increased (39–50%) and that by 10 mM-citrate decreased (25–13%) when the pyruvate concentration was decreased from 50 to 10 mM (Table 5). A tenfold decrease in the $MnCl_2$ concentration (0·33–0·033 mM) lowered the inhibition by 0·167 mM-fluorocitrate from 39 to 28% but increased that by 10 mM-citrate from 25 to 44%.

DISCUSSION

The data show that the initial rate of the synthesis of malate by malic enzyme is inhibited by the same carboxylic acids as that of the decarboxylation of malate (see Table 6). However, the concentration required for 50% inhibition was, in most cases, different for the two directions of the reaction. Oxaloacetate was the only inhibitor of malate decarboxylation effective at a concentration less than that of the substrate. In the reverse direction, not only oxaloacetate, but also malonate, tartronate and fluorocitrate inhibited at concentrations less than that of the substrate. Fluorocitrate was the only compound tested which inhibited malate synthesis at concentrations less than those at which it inhibited malate decarboxylation. The three C_3-dicarboxylic acids (malonic acid, tartronic acid and mesoxalic acid) inhibited the decarboxylation reaction to approximately the same extent,

but they were inhibitory to malate synthesis at widely differing concentrations. Also, in this latter direction, it appeared that the mechanism of the inhibition was different since the malonate inhibition was partially competitive, the tartronate inhibition non-competitive and that by mesoxalate partially uncompetitive. No explanation can be offered for these findings.

The decarboxylation of malate is not very specific in cation requirements, Mn^{2+}, Mg^{2+} and Co^{2+} ions, all activating at the same concentration (Stickland, 1959). For malate synthesis Mn^{2+} ions were much more efficient than the others, giving a maximum rate at 1% of the concentration of Mg^{2+} or Co^{2+} ions required. The Mn^{2+} ion (unhydrated) has a radius of 0·80 Å; the radii of the bivalent Mg^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+} ions are 0·65–0·74 Å (values of Pauling, as quoted in Hodgman, 1950). The radius of the Mn^{2+} ion is thus greater than that of all the other ions whose effects on malate synthesis were studied, and this ion might fit the enzyme–substrate complex better. The inhibition by Zn^{2+} and Cu^{2+} ions is presumably due to their blocking the active centre.

The non-enzymic removal of TPNH formed by reduction of TPN^+ with dithionite is brought about only by metals that are members of the first transition period of the periodic table. Thus these metals all have a variable valency. The disappearance of TPNH is probably due to its oxidation, the ions acting catalytically. It is not clear why this oxidation should be affected by bicarbonate.

The K_m for pyruvate (2×10^{-2} M) is very high. The pyruvate concentration in rat liver is approximately 0·6 mM (as calculated from the data of Frohman, Orten & Smith, 1951). Assuming it is about the same in pigeon liver, the malic enzyme is far from saturated with pyruvate. However, since the K_m is so much higher than the pyruvate concentration, the rate of malate synthesis will be approximately proportional to the pyruvate concentration.

SUMMARY

1. The synthesis of malate from pyruvate and bicarbonate by the malic enzyme of pigeon liver has been studied.

2. Malate synthesis was inhibited by carbon dioxide when the carbon dioxide pressure was above about 0·45 atmosphere. The apparent Michaelis constant for pyruvate was very high.

3. Bivalent metal ions were essential for the reaction. The same rate was given by 0·033 mM-Mn^{2+} as by 3·3 mM-Mg^{2+} or Co^{2+} ions. Zn^{2+} and Cu^{2+} ions were inhibitory.

4. In the presence of 10 mM-pyruvate, 40–50% inhibition was caused by tartronate (0·1–0·01 mM), fluorocitrate (0·1 mM), malonate (mM), L-malate (mM), oxaloacetate (1–10 mM), D- and L-lactate, DL-α-hydroxybutyrate and mesoxalate (all 20 mM). *meso*Tartrate and citrate were weaker inhibitors. Propionate and DL-β-hydroxybutyrate were not inhibitory.

5. Triphosphopyridine nucleotide which had been reduced by dithionite was non-enzymically oxidized in the presence of Mn^{2+}, Co^{2+}, Ni^{2+} or Cu^{2+} ions. Enzymically reduced triphosphopyridine nucleotide did not show this phenomenon unless sulphite was added. The oxidation in the presence of Cu^{2+} ions was inhibited by bicarbonate; that by the other ions was increased by bicarbonate.

REFERENCES

Beisenherz, G., Boltze, H. J., Bücher, T., Czök, R., Garbade, K-H., Meyer-Arendt, E. & Pfleiderer, G. (1953). *Z. Naturf.* **8** b, 555.

Dixon, M. (1953). *Biochem. J.* **55**, 170.

Ebersole, E. R., Guttentag, C. & Wilson, P. W. (1944). *Arch. Biochem.* **3**, 399.

Frohman, C. E., Orten, J. M. & Smith, A. H. (1951). *J. biol. Chem.* **193**, 803.

Hodgman, C. D. (1950). In *Handbook of Chemistry and Physics*, 32nd ed., p. 2808. Cleveland: Chemical Rubber Publishing Co.

Kornberg, A. & Horecker, B. L. (1953). *Biochem. Prep.* **3**, 24.

Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.

Rackis, J. J. & Kalnitsky, G. (1957). *J. biol. Chem.* **225**, 751.

Stickland, R. G. (1959). *Biochem. J.* **73**, 646.

Umbreit, W. W. (1949). In *Manometric Techniques and Tissue Metabolism*, 2nd ed., p. 27. Minneapolis: Burgess Publishing Co.

Some Properties of Oxaloacetate-Synthesizing Enzyme

By R. G. STICKLAND*
Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, University of Oxford

(*Received* 14 *April* 1959)

Utter & Kurahashi (1954; see also Kurahashi, Pennington & Utter, 1957) discovered an enzyme in pigeon liver which catalyses the reaction:

$$\text{Phosphopyruvate} + (\text{IDP or GDP}) + CO_2 \xrightleftharpoons{Mn^{2+}} \text{oxaloacetate} + (\text{ITP or GTP})$$

where IDP and ITP stand for inosine di- and tri-phosphate and GDP and GTP stand for guanosine di- and tri-phosphate. They refer to it as 'oxaloacetate-synthesizing enzyme' (Utter & Kurahashi, 1955). Relatively little is known of the kinetics of the enzyme and of the effects of inhibitors. The enzyme is assumed to play a role in the synthesis of glycogen from pyruvate (see Krebs, 1954; Hiatt, Goldstein, Lareau & Horecker, 1958), and possibly in the supply of oxaloacetate in liver. For the reasons stated by Stickland (1959*a*) it was of interest to investigate the inhibition of the enzyme by those carboxylic acids which are known to cause an accumulation of acetoacetate in respiring liver.

EXPERIMENTAL

Preparation of enzyme. Except for the first stages, the method used was that of Utter & Kurahashi (1955). From the observations of Bartley & Avi-Dor (1955), it was thought that an initial purification might be obtained by preparing an extract of mitochondria instead of an acetone-dried powder of liver as was used by Utter & Kurahashi. Kurahashi *et al.* (1957) have prepared the enzyme from an acetone-dried powder of chicken-liver mitochondria. All procedures were carried out at 0° unless otherwise stated.

The livers of 20 pigeons were removed and immediately placed in ice–water mixture. The livers (180 g.) were homogenized (in a stainless-steel homogenizer of the Potter–Elvehjem type) in 180 ml. of 0·9% KCl and spun at 700 *g* for 2 min., this removing unbroken cells, nuclei and cell debris. The mitochondria were then separated from the supernatant fraction by centrifuging for 15 min. at 3000 *g*. The mitochondria were suspended in 5 vol. (550 ml.) of water in a Waring Blendor and blended for eight periods of 2 min. each during 1 hr. To the disrupted mitochondria was added sufficient potassium phosphate buffer (0·25 M, pH 7·8) to bring the phosphate concentration to mM. This mixture was then shaken at 38° for 10 min. and the residue spun off (20 min., 10 000 *g*). The supernatant was used for step 2 of the procedure of Utter & Kurahashi (1955).

The purification was continued to the end of the calcium phosphate gel stage. This enzyme fraction was diluted 10- to 20-fold when required and 0·1 ml. of the diluted solution was used for each experiment.

The protein content of the concentrated enzyme solution was 7·2 mg./ml., as estimated by the biuret method, bovine serum albumin (The Armour Laboratories) being used as standard.

Special chemicals. The disodium salt of adenosine triphosphate (ATP) and the Ba salt of adenosine diphosphate (ADP) were obtained from Schwarz Laboratories Inc., New York 17, N.Y., U.S.A. As assayed by the method of Krebs & Hems (1953), the ATP was 86% pure and no phosphate-containing impurities could be detected in the ADP. Inosine diphosphate (IDP) was prepared from ADP by the method of Kleinzeller (1942) for the conversion of ATP into ITP. Phosphopyruvate (Ba–Ag salt) was prepared by the method of Ohlmeyer (1951). The Ba or Ba–Ag salts of these compounds were converted into the Na or K salts by shaking them at 0° with an excess of Dowex-50 resin (25–40 mesh) in the appropriate form.

ATP, ADP, inosine triphosphate (ITP) and IDP were assayed by the method of Krebs & Hems (1953).

Disodium fluoromalate (2-fluoro-3-hydroxysuccinate) and fluorocitrate (triethyl ester) were kindly supplied by Dr P. W. Kent. The triethylfluorocitrate was hydrolysed by heating with the theoretical amount of NaOH at 100° for 1 hr.

Measurement of enzyme activity. The activity of the enzyme was measured by determining colorimetrically the amount of oxaloacetate formed from sodium phosphoenolpyruvate in unit time.

The reaction mixture, which had a total volume of 1·5 ml., contained 2-amino-2-hydroxymethylpropane-1:3-diol chloride (tris) buffer, pH 7·4 at 30°, sodium phosphoenolpyruvate, sodium salt of IDP, $NaHCO_3$, enzyme, activators and inhibitors as given in the details of the individual experiments. The usual concentrations were as follows: tris (0·1 M), phosphoenolpyruvate (mM), IDP (0·4 mM), $NaHCO_3$ (16 mM) and $MnCl_2$ (mM). The stock enzyme solution (0·05–0·07 ml.) was diluted to 1 ml. as required and 0·1 ml. of this solution was placed in the hollow stopper of a Thunberg tube. All other constituents of the reaction medium were placed in the Thunberg tubes.

The hollow stopper containing the enzyme was cooled in ice for 5 min., and then placed in the tube, which was evacuated and filled with $CO_2 + N_2$ (5:95) three times. The tube was placed in a bath at 30° and allowed to equilibrate for 5–7 min., when the reaction was started by mixing the tube and stopper contents. After a suitable time interval (usually 5 min.) the reaction was stopped by the addition of 0·5 ml. of 25% (w/v) trichloroacetic acid.

* Present address: John Innes Horticultural Institution, Bayfordbury, Hertford, Herts.

In some experiments the $NaHCO_3$ concentration was varied. In order to maintain the pH at 7·4 the percentage of CO_2 in the gas phase was changed according to the data given by Umbreit (1949).

The amount of oxaloacetate present in the trichloroacetic acid extract was measured as given below. It was not found necessary to remove the precipitated protein as the small amount present (less than 0·05 mg.) did not interfere with the estimation.

Experiments on the removal of added oxaloacetate by the enzyme were conducted in a similar manner. Bicarbonate was omitted and the gas phase was N_2. Other details are given with the individual experiments.

Estimation of oxaloacetate. Oxaloacetate was measured by a modification of the method of Kalnitsky & Tapley (1958). Since neither ascorbic acid nor acetoacetate was present here, the Kalnitsky & Tapley method was simplified by omitting the addition of $NaNO_2$ and the controls used to determine the acetoacetate. The procedure was as follows.

Immediately after the reaction was stopped by the addition of trichloroacetic acid, the tubes and contents (total volume 2 ml.) were placed in ice. When the tubes had been in ice for 5 min., 1·2 ml. of cold (5°) 1·66 M-sodium acetate was added, followed by 3 ml. of the diazo reagent of Walker (1954). The solution was incubated in a water bath at 25° for 20 min., placed in ice for 5 min. and 4 ml. of ethyl acetate, followed by 2 ml. of 5 N-HCl, were added. The tube was shaken vigorously within $7\frac{1}{2}$ sec. of adding the HCl and the shaking was continued for 15 sec. The extinction of the ethyl acetate layer was read at 450 mμ. At this wavelength, 100 μm-moles of oxaloacetate gave an extinction of 0·690 in 1 cm. cells. Malonate, fluoromalate, fluorocitrate and citrate gave colours with this procedure, 1 μmole of the compounds giving respectively as much colour as 140, 2·5, 21 and 35 μm-moles of oxaloacetate. The phosphopyruvate preparation used gave about 0·2% of the colour given by an equal concentration of oxaloacetate. Under the test conditions none of the other compounds interfered with the estimation at the highest concentrations at which they were used.

Analysis of kinetics. Values of the apparent Michaelis constant (K_m) for one set of conditions only were obtained by the method of Lineweaver & Burk (1934) and values of K_i (the dissociation constant of the enzyme–inhibitor complex) by the method of Dixon (1953). The terms v, [S] and [I] refer respectively to the initial velocity and substrate and inhibitor concentrations.

RESULTS

Time course of formation of oxaloacetate from phosphopyruvate. Preliminary experiments showed that the rate was linear for at least 6 min. under the test conditions (Fig. 1). In subsequent experiments an incubation period of 5 min. was taken as a convenient standard time.

Effect of substrate concentration. The rate of oxaloacetate formation increased with the concentration of phosphopyruvate but no maximum rate was reached by mM-phosphopyruvate (Fig. 2). When the IDP concentration was varied in the presence of mM-phosphopyruvate the rate reached a maximum at mM-IDP concentration. The values of the apparent K_m for these two substrates were 2×10^{-4} M for phosphopyruvate and 4×10^{-5} M for IDP (Table 1).

When IDP was replaced by ADP the rate fell to about one-tenth and this remaining activity was

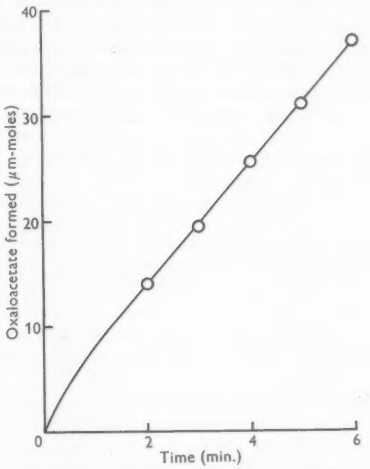


Fig. 1. Formation of oxaloacetate with time. Each tube contained, in a total volume of 1·5 ml., tris buffer, pH 7·4 (0·1 M), $NaHCO_3$ (16·5 mM), $MnCl_2$ (mM), phosphoenolpyruvate (mM), IDP (0·4 mM) and enzyme (0·05 ml.). Gas phase, $CO_2 + N_2$ (5:95).

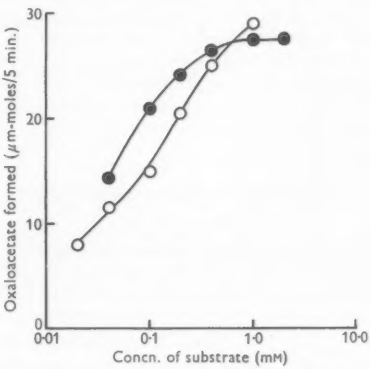


Fig. 2. Effect of phosphoenolpyruvate and IDP concentrations. Tube contents were as given in Fig. 1, except for the component which was varied. ●, Effect of IDP concentration in the presence of mM-phosphoenolpyruvate; ○, effect of phosphoenolpyruvate concentration in the presence of 0·4 mM-IDP. In the absence of either phosphoenolpyruvate or IDP 0·7 μm-mole of oxaloacetate was formed in 5 min.

probably due to traces of IDP or guanosine diphosphate and to nucleoside diphosphokinase, the presence of which was shown by the experiment recorded in Table 2: when ATP or IDP was incubated alone, no other nucleotides were formed, but when added together ITP and ADP appeared.

In the absence of added CO_2–bicarbonate very little oxaloacetate was formed (Fig. 3). Increase in the CO_2–bicarbonate concentration resulted in an increase in the rate of oxaloacetate formation. A maximum rate was reached at about 30 % of CO_2 in the gas phase (0·1 M-HCO_3^- ion). Higher concentrations of CO_2–bicarbonate produced inhibition (about 50 % with a gas phase of 100 % CO_2). This may be due to the high ionic strength, since NaCl also inhibited (see below). The apparent K_m for CO_2 was 2 mM-CO_2 in solution or 25 mM-bicarbonate under the conditions used.

Table 1. *Values of K_m and K_i*

Values of the apparent K_m, for one set of conditions only, were obtained by the method of Lineweaver & Burk (1934) and values of K_i by the method of Dixon (1953).

	K_m (M)
Phosphoenolpyruvate	2×10^{-4}
IDP	4×10^{-5}
CO_2	2×10^{-3}
Mn^{2+}	1×10^{-4}
	K_i (M)
Tartronate	2×10^{-4}

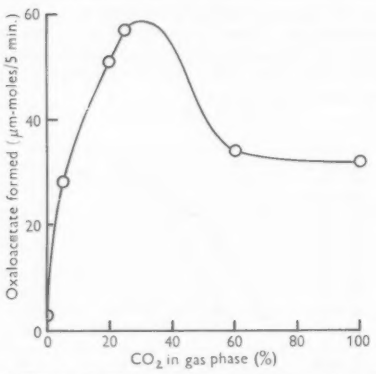


Fig. 3. Effect of CO_2 on oxaloacetate formation. Tube contents were as given in Fig. 1, except for the $NaHCO_3$, which was varied with the CO_2 to keep the pH at 7·4.

Effect of cations. Mn^{2+} ions were essential for the reaction to proceed at maximum rate (Table 3, Expt. 1). The rate of oxaloacetate formation with Mg^{2+} and Co^{2+} ions was about 10 % of that with an equal concentration (mM) of Mn^{2+} ions. A tenfold increase in the Mg^{2+} ion concentration had no effect. Little or no oxaloacetate was formed in the presence of Ni^{2+}, Cu^{2+}, Zn^{2+} or Ca^{2+} ions.

The rate of oxaloacetate formation increased with the $MnCl_2$ concentration up to a maximum of mM-$MnCl_2$ (Fig. 4). Higher concentrations were inhibitory; at 33 mM-$MnCl_2$ the rate of oxaloacetate formation was only 20 % of the maximum rate. The apparent K_m for Mn^{2+} ions was 0·1 mM with reference to the overall Mn^{2+} ion concentration.

Mn^{2+} ions are very tightly chelated by phosphopyruvate (binding constant 560 l./mole; Wold & Ballou, 1957). With mM-$MnCl_2$ and mM-phosphopyruvate the concentration of free Mn^{2+} ions will be low, but when the amount of added $MnCl_2$ exceeded that of phosphopyruvate free Mn^{2+} ions are present in appreciable quantities and these ions catalyse the decarboxylation of oxaloacetate (Krebs, 1942). The apparent inhibition of oxaloacetate formation might be due to the decarboxylation of oxaloacetate by Mn^{2+} ions and this was tested by incubating oxaloacetate with enzyme and varying amounts of $MnCl_2$. In the absence of $MnCl_2$ there was a small decomposition (3 %) of oxaloacetate in 5 min. (Table 4), but this was increased to 7 % by mM-$MnCl_2$ and to 37 % by 10 mM-$MnCl_2$. The addition of mM-phosphopyruvate in the presence of mM-$MnCl_2$ decreased the decomposition of oxaloacetate to 3 %.

When added in the presence of $MnCl_2$, Co^{2+}, Cu^{2+} and Zn^{2+} ions inhibited the synthesis of oxaloacetate but Mg^{2+} and Ca^{2+} ions did not (Table 3, Expt. 2). The total concentration of bivalent cations in these experiments was 2 mM, a concentration at which Mn^{2+} ions inhibited about 10 % (Fig. 4).

Potassium chloride inhibited oxaloacetate formation at concentrations of 10 mM and above

Table 2. *Evidence for the presence of nucleoside diphosphokinase*

Tubes contained tris buffer, pH 7·4 (0·1 M), $MnCl_2$ (3·3 mM), enzyme (0·05 ml.) and additions as shown, in a total volume of 0·9 ml., and were incubated for 15 min. at 30°. Reaction was stopped with 0·1 ml. of 30 % (w/v) trichloroacetic acid.

	Nucleotides present (μmoles)			
Additions	ATP	ADP	ITP	IDP
ATP + IDP, unincubated	1·63	0	0	1·67
ATP, incubated	1·60	0	0	0
IDP, incubated	0	0	0	1·60
ATP + IDP, incubated	0·98	0·72	0·93	0·69

(Fig. 4). There was 90% inhibition by 0·1 M-KCl. The curve shown in Fig. 4 was obtained in the presence of approximately 0·02 M-Na^+ ion (added as the cation equivalent of bicarbonate, phosphopyruvate and IDP). Sodium chloride inhibited at concentrations of 0·1 M (14%) and 0·22 M (33%).

Table 3. *Effect of cations on oxaloacetate formation*

Each tube contained 0·1 ml. of enzyme and metal salts (mM) as stated. Other additions (except $MnCl_2$) were as given in Fig. 1. In Expt. 2 mM-$MnCl_2$ was present in all tubes in addition to the salt stated in the Table.

Expt. no.	Metal salt added	Oxaloacetate formed (μm-moles/5 min.)
1	None	0·7
	$MnCl_2$	27·8
	$MgCl_2$	2·5
	$CoCl_2$	3·9
	$NiSO_4$	0
	$CuSO_4$	1·6
	$ZnSO_4$	1·0
	$CaCl_2$	0
		Decrease in the rate compared with that for $MnCl_2$ alone (%)
2	$MgCl_2$	0
	$CoCl_2$	30
	$NiSO_4$	13
	$CuSO_4$	37
	$ZnSO_4$	90
	$CaCl_2$	4

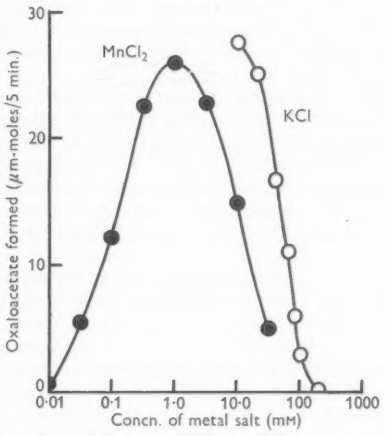


Fig. 4. Effect of $MnCl_2$ and KCl on oxaloacetate formation. Tube contents were as given in Fig. 1, except for the component varied as above. In the absence of $MnCl_2$, 0·3 μm-mole of oxaloacetate was formed in 5 min. The KCl curve was obtained in the presence of approx. 0·02 M-Na^+ and mM-Mn^{2+} ions. The rate in the absence of added KCl was 29·1 μm-moles of oxaloacetate formed in 5 min.

When the bicarbonate, phosphopyruvate and IDP were added as potassium instead of sodium salts, the inhibition by added KCl was exactly the same if the total K^+ ion concentration was taken into account.

Effect of carboxylic acids. L-Malate and *meso*-tartrate (10 mM) both had a slight inhibitory effect (10 and 14% respectively) with mM-phosphopyruvate as substrate. A threefold increase in the $MnCl_2$ concentration brought about a considerable reduction (to 4%) in this inhibition. The action of these inhibitors might thus have been due to the chelation of cations.

At a concentration of 10 mM, tartronate was a much stronger inhibitor than malate, giving about 70% inhibition. However, at tartronate concentrations above mM, plots of $1/v$ against [I] were non-linear. At lower concentrations, plots of $1/v$ against [I] were linear (see Fig. 5) and gave a value for K_i of 2×10^{-4} M. The plots also showed that tartronate was a competitive inhibitor: a tartronate concentration of 0·8 mM inhibited 30% at mM-phosphopyruvate and 52% at 0·4 mM-phosphopyruvate (Table 5).

Oxalate also inhibited the formation of oxaloacetate. The inhibition was intermediate between purely competitive and purely non-competitive inhibition. Thus 0·2 mM-oxalate inhibited 16% at mM-phosphopyruvate and 32% at 0·1 mM-phosphopyruvate. However, 0·8 mM-oxalate inhibited 54% whether the phosphopyruvate concentration was 0·4 mM or 0·1 mM (Table 5). No value for K_i could be obtained because plots of $1/v$ against [I] and of $1/v$ against $1/[S]$ were non-linear (Fig. 5).

The effect of mesoxalate had to be studied with $MgCl_2$ to activate the enzyme instead of $MnCl_2$, owing to the formation of an insoluble compound between $MnCl_2$ and mesoxalate. Since the activity of the enzyme was very low in the presence of $MgCl_2$ (Table 3), the effect of mesoxalate could not be

Table 4. *Effect of enzyme on oxaloacetate*

Each tube contained tris buffer, pH 7·4 (0·1 M), enzyme (0·1 ml.) and additions as stated below, in a total volume of 1·5 ml. Incubations were for 5 min. at 30°. Oxaloacetate concentration was 33 μM (50 μm-moles/tube) and phosphopyruvate concentration was mM.

Additions	Concn. of $MnCl_2$ (mM)	Oxaloacetate present (μm-moles)
Oxaloacetate	0	49·0*
Oxaloacetate	0	47·6
Oxaloacetate	1	44·6
Oxaloacetate	10	30·2
Oxaloacetate + phosphopyruvate	1	47·6
Phosphopyruvate	1	0·4

* Not incubated.

studied in detail. A concentration of 10 mM-mesoxalate (with mM-phosphopyruvate) inhibited 52%.

Malonate gave a colour in the oxaloacetate estimation. As a result, 0·2 mM was the highest concentration at which it could be used without giving a blank reading which was too high. At this concentration, and in the presence of an equal concentration of phosphopyruvate, malonate inhibited 15%. If the $MnCl_2$ concentration was increased from mM to 3 mM, the inhibition was reduced to 8%.

Fluoromalate, fluorocitrate and citrate also interfered with the oxaloacetate estimation. Under the same conditions as for Fig. 1, but with 0·1 mM-phosphopyruvate, 10 mM-fluoromalate gave complete inhibition but 0·1 mM-fluoromalate gave almost none. With mM-fluoromalate, a threefold increase in phosphopyruvate concentration had almost no effect on the inhibition, which was reduced from 22 to 20%. The figure of 22% was not altered if the $MnCl_2$ concentration was increased from mM to 3 mM.

Fluorocitrate and citrate both gave 21% inhibition with both inhibitor and substrate at a concentration of mM.

Of these weaker inhibitors, malonate, fluorocitrate and *meso*tartrate had no effect on the formation of phosphopyruvate from oxaloacetate (Stickland, unpublished work). The effect of citrate and L-malate on this latter reaction was not tried.

The figures given for the small inhibitions by malonate, fluorocitrate and citrate are not very reliable owing to the large blank which these compounds gave in the oxaloacetate estimation.

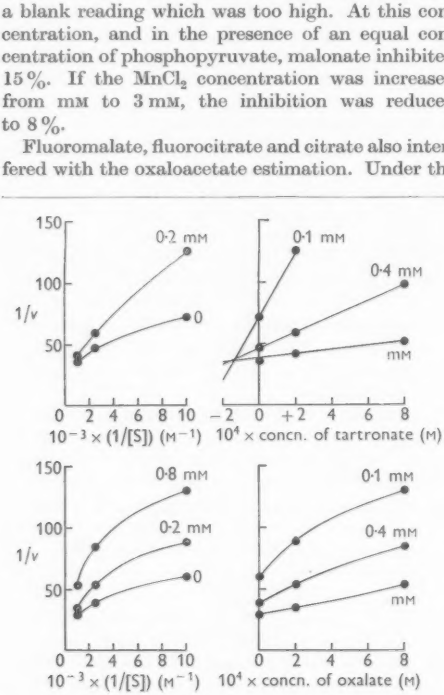


Fig. 5. Effect of tartronate and oxalate on oxaloacetate formation. Top graphs are for tartronate; bottom graphs are for oxalate. Left-hand graphs are plots of $1/v$ against $1/[S]$; the figures on the graph indicate the inhibitor concentration. Right-hand graphs are plots of $1/v$ against inhibitor concentration; the figures on the graph indicate the substrate concentration. v, Initial velocity as μmoles of oxaloacetate formed in 5 min.; [S], substrate concentration.

Table 5. *Effect of tartronate and oxalate on oxaloacetate formation*

Tube contents were as given in Fig. 1, except where stated below.

Concn. of phospho-pyruvate (mM)	Inhibition (%)			
	Tartronate		Oxalate	
	0·8 mM	0·2 mM	0·8 mM	0·2 mM
1	30	15	46	16
0·4	52	20	54	28
0·1	—	42	54	32

DISCUSSION

Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+} ions catalyse the non-enzymic decarboxylation of oxaloacetate to a greater extent than do Mn^{2+} ions (Speck, 1948), and it is possible that they decrease oxaloacetate formation not by inhibiting the enzyme but by decomposing the oxaloacetate once it is formed. Support for this assumption is that Mg^{2+} and Ca^{2+} ions, which are poorer catalysts than are Mn^{2+} ions for the decarboxylation of oxaloacetate, caused little decrease in the formation of oxaloacetate (Table 3, Expt. 2).

Boyer, Lardy & Phillips (1943) found that pyruvic kinase was activated by K^+ ion in concentrations up to 0·1 M, but that higher concentrations were inhibitory. In contrast, the oxaloacetate-synthesizing enzyme is not activated by K^+ ions, but concentrations greater than 10 mM inhibited (Fig. 4). Both enzymes are inhibited by 0·1 M-Na^+ ion.

The K^+ ion concentration in the water of the cells of dog liver is about 0·15 M and the Na^+ ion concentration about 3 mM (see Harris, 1956). This Na^+ ion concentration is not sufficient to cause inhibition of pyruvic kinase or oxaloacetate-synthesizing enzyme, but marked inhibition would be expected by 0·15 M-K^+ ion. However, it is possible that some of the K^+ ion is bound and the concentration of K^+ ion free to influence the activity of these two enzymes may be less than 0·15 M. Relatively small variations in K^+ ion concentration would affect the activity of both these enzymes.

Six ketogenic substances have been found to inhibit malic enzyme (Stickland, 1959*a*, *b*). These are malonate, tartronate, mesoxalate, oxalate,

*meso*tartrate and fluorocitrate. In addition Gal (1958) has found fluoromalate to inhibit malic enzyme. Of these substances only tartronate, mesoxalate, oxalate and fluoromalate give appreciable inhibition of oxaloacetate-synthesizing enzyme.

The ketogenic effect of tartronate, mesoxalate, oxalate and fluoromalate could be accounted for by the assumption that it is due to an inhibition of the formation of oxaloacetate via either malic enzyme or oxaloacetate-synthesizing enzyme. However, the ketogenic effect of fluorocitrate and malonate could be explained by inhibition of malic enzyme but not of oxaloacetate-synthesizing enzyme. In addition, malonate and oxalate inhibit succinic and malic dehydrogenases (Quastel & Wooldridge, 1928; Green, 1936; Lardy, 1949). Fluorocitrate inhibits aconitase (Morrison & Peters, 1954). Davies & Kun (1957) found that D- and *meso*-tartrate, tatronate and mesoxalate inhibit the oxidation of malate by malic dehydrogenase. It is therefore possible that the ketogenic action of these compounds is due to inhibition of some stages of the citric acid cycle and thus to a decrease in the formation of oxaloacetate.

SUMMARY

1. The effect of inhibitors on the formation of oxaloacetate from phosphopyruvate by the oxaloacetate-synthesizing enzyme of pigeon liver has been studied, and data have been obtained on the kinetics of the reaction.

2. Under the conditions studied, the values of the apparent K_m were as follows: phosphopyruvate (2×10^{-4}M), inosine diphosphate (4×10^{-5}M), carbon dioxide (2×10^{-3}M) and Mn^{2+} ion (10^{-4}M).

3. It is confirmed (Utter & Kurahashi, 1954) that for the maximum rate of formation of oxaloacetate added Mn^{2+} ions were required; Mg^{2+} and Co^{2+} ions were almost inactive. High concentrations (10 mM) of Mn^{2+} ions decreased the oxaloacetate yield, probably owing to the non-enzymic decarboxylation of the oxaloacetate. Co^{2+}, Ni^{2+} and Cu^{2+} ions also decreased the yield.

4. Potassium chloride (0·1M) inhibited about 90%. Sodium chloride at the same concentration inhibited 14%.

5. Tartronate and oxalate inhibited the reaction about 50% at a concentration twice that of phosphopyruvate. The inhibition was partially competitive and partially non-competitive. A value for K_i of 2×10^{-4}M was obtained graphically for tartronate. Mesoxalate (10 mM) inhibited 52% with mM-phosphopyruvate and fluoromalate (mM) 22% with 0·1 mM-phosphopyruvate. There was less inhibition (approx. 15%) by L-malate, *meso*tartrate, malonate, fluorocitrate and citrate.

6. The ketogenic effect of tartronate, mesoxalate, oxalate and fluoromalate could be accounted for by the assumption that it is due to an inhibition of the formation of oxaloacetate via either malic enzyme or oxaloacetate-synthesizing enzyme. However, the ketogenic effect of fluorocitrate and malonate could possibly be explained by inhibition of malic enzyme but not of oxaloacetate-synthesizing enzyme.

REFERENCES

Bartley, W. & Avi-Dor, Y. (1955). *Biochem. J.* **59**, 194.
Boyer, P. D., Lardy, H. A. & Phillips, P. H. (1943). *J. biol. Chem.* **149**, 529.
Davies, D. D. & Kun, E. (1957). *Biochem. J.* **66**, 307.
Dixon, M. (1953). *Biochem. J.* **55**, 170.
Gal, E. M. (1958). *Arch. Biochem. Biophys.* **73**, 279.
Green, D. E. (1936). *Biochem. J.* **30**, 2095.
Harris, E. J. (1956). *Transport and Accumulation in Biological Systems*, p. 221. London: Butterworths.
Hiatt, H. H., Goldstein, M., Lareau, J. & Horecker, B. L. (1958). *J. biol. Chem.* **231**, 303.
Kalnitsky, G. & Tapley, D. F. (1958). *Biochem. J.* **70**, 28.
Kleinzeller, A. (1942). *Biochem. J.* **36**, 729.
Krebs, H. A. (1942). *Biochem. J.* **36**, 303.
Krebs, H. A. (1954). *Johns Hopk. Hosp. Bull.* **95**, 19.
Krebs, H. A. & Hems, R. (1953). *Biochim. biophys. Acta*, **12**, 172.
Kurahashi, K., Pennington, R. J. & Utter, M. F. (1957). *J. biol. Chem.* **226**, 1059.
Lardy, H. A. (1949). In *Respiratory Enzymes*, revised ed., p. 119. Minneapolis: Burgess Publishing Co.
Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
Morrison, J. F. & Peters, R. A. (1954). *Biochem. J.* **58**, 473.
Ohlmeyer, P. (1951). *J. biol. Chem.* **190**, 21.
Quastel, J. H. & Wooldridge, W. R. (1928). *Biochem. J.* **22**, 689.
Speck, J. F. (1948). *J. biol. Chem.* **178**, 315.
Stickland, R. G. (1959*a*). *Biochem. J.* **73**, 646.
Stickland, R. G. (1959*b*). *Biochem. J.* **73**, 654.
Umbreit, W. W. (1949). In *Manometric Techniques and Tissue Metabolism*, 2nd ed., p. 27. Minneapolis: Burgess Publishing Co.
Utter, M. F. & Kurahashi, K. (1954). *J. biol. Chem.* **207**, 787, 803, 821.
Utter, M. F. & Kurahashi, K. (1955). In *Methods in Enzymology*, vol. 1, p. 758. New York: Academic Press Inc.
Walker, P. G. (1954). *Biochem. J.* **58**, 699.
Wold, F. & Ballou, C. E. (1957). *J. biol. Chem.* **227**, 301.

Studies on Sulphatases

26. ARYLSULPHATASE ACTIVITY IN THE DIGESTIVE JUICE AND DIGESTIVE GLAND OF *HELIX POMATIA**

BY K. S. DODGSON AND GILLIAN M. POWELL
Department of Biochemistry, University of Wales, Newport Road, Cardiff

(*Received* 24 *April* 1959)

During the course of studies on arylsulphatases in these Laboratories evidence has accumulated which suggests that these enzymes can be classified into two types. The type I enzymes are particularly active towards simple arylsulphates such as potassium *p*-nitrophenyl sulphate and potassium *p*-acetylphenyl sulphate. They are inhibited by cyanide but not by phosphate and fluoride. In contrast the type II enzymes show relatively greater activity towards more complex arylsulphates such as dipotassium 2-hydroxy-5-nitrophenyl sulphate, and their behaviour towards cyanide, phosphate and fluoride is the reverse of that shown by the type I enzymes.

Recently arylsulphatases from a variety of organisms have been investigated in attempts to provide additional evidence for the existence of two distinct types of enzyme. One of the enzymes which was of particular interest was that present in the edible snail, *Helix pomatia*. This arylsulphatase has previously been studied in some detail by Jarrige & Henry (1952) and by Dastugue, Bastide & Tronche (1957), but from their results it is not possible to deduce with certainty whether the enzyme can be classified as type I or II. In particular these two groups of workers differed in their findings about the effect of phosphate on enzyme activity. Jarrige & Henry (1952), using potassium *p*-nitrophenyl sulphate as the substrate, reported only 48% inhibition of enzyme activity by 0·1M-phosphate, whereas Dastugue *et al.* (1957), using sodium 2-carboxyphenyl sulphate as substrate, obtained 98% inhibition with 0·01M-phosphate.

In an attempt to resolve these and other discrepancies *Helix* arylsulphatase has been re-examined in these Laboratories. Initially, digestive juice obtained from the crop of the organism was used as the enzyme source but it was subsequently necessary (for reasons outlined in the text) to abandon this in favour of extracts of the digestive gland (hepatopancreas). The results described in this and the next paper show that, in certain respects, the *Helix* enzyme appears to be unlike any arylsulphatase previously described.

* Part 25: Spencer (1959).

MATERIALS AND METHODS

Snails. These were obtained from L. Haig, Beambrook, Newdigate, Surrey and were starved for 4 days before use. Occasionally during the winter months it was necessary to use hibernating snails. In such cases the organisms were kept in a humid atmosphere at a temperature of about 25° for a week, when most of them became active and began to feed. In general, the arylsulphatase activity of hibernating snails was appreciably lower than that of organisms collected in spring and summer.

Substrates. Potassium *p*-nitrophenyl sulphate (NPS) was prepared by the method of Burkhardt & Lapworth (1926) and dipotassium 2-hydroxy-5-nitrophenyl sulphate (NCS) by the method of Roy (1953) modified as described by Dodgson & Spencer (1956).

Determination of enzyme activity. Arylsulphatase activity towards NPS and NCS was measured by spectrophotometric estimation of the anionic form of the liberated phenolic moiety, at the appropriate wavelength of maximum absorption, after the addition of alkali to the incubation mixture (see Dodgson & Spencer, 1957). With crude digestive-gland extracts when NCS was the substrate the colour of the anionic form of the liberated 4-nitrocatechol tended to fade rather rapidly. This could be eliminated by developing the colour with the alkaline quinol–sodium sulphite reagent described by Roy (1953). Except where otherwise stated, incubation of enzyme (0·2 ml.) and substrate (0·2 ml.) was for 30 min. (for the digestive-juice enzyme) or 15 min. (digestive-gland extract) at 38° in the presence of 0·25M-acetic acid–sodium acetate buffer at the appropriate pH or 0·25M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) which had been adjusted to the required pH with acetic acid. On some occasions acetic acid–sodium acetate buffer was used at pH values where its buffering power was low. However, separate experiments, in which the pH of the incubation mixture was checked at the beginning and the end of the incubation period, showed that no significant change in pH had occurred. Preliminary experiments were made in order to establish the concentration of enzyme appropriate to the range of accuracy of the assay method.

EXPERIMENTAL AND RESULTS

Experiments with digestive juice

Preliminary experiments were made on a sample of the digestive juice which had been stored at 0° for several months. In later experiments digestive

juice was expressed from the crop of freshly dissected snails. Results obtained with both fresh and stored juice were substantially the same. The arylsulphatase activity of the juice is extremely high (cf. Jarrige & Henry, 1952) and the preparations had to be diluted approximately 10 000-fold to bring the activity within the range of the assay methods.

Effect of pH *and substrate concentration.* Jarrige & Henry (1952) have reported that *Helix* arylsulphatase shows maximum activity at pH 5·2 and at a substrate (NPS) concentration of 0·5 mM. Attempts were made to confirm these findings with both dialysed and non-dialysed samples of the juice. During the course of these experiments it became apparent that the optimum pH of the enzyme towards NPS varied appreciably with the concentration of substrate used and, similarly, the optimum substrate concentration varied with pH. Fig. 1 shows the effect of increasing substrate concentration on the optimum pH of a fixed amount of enzyme. There is a gradual shift in the optimum pH from 6·0 in the presence of 0·5 mM-NPS to 7·3 in the presence of 15 mM-NPS. Further increase in substrate concentration to 30 mM did not result in a further shift in pH optimum. No attempt was made to determine pH optima at NPS concentrations below 0·5 mM because a high proportion (> 40 %) of the substrate present in the incubation mixture would have been hydrolysed before suitable spectrophotometric readings could be made. Separate experiments showed that at pH 6·0 maximum enzyme activity was obtained with 6 mM-NPS, whereas at pH 7·0 and 7·3 the corresponding values were 12 and 15 mM. Similar results were obtained with dialysed and non-dialysed digestive juice.

Fig. 2 shows that similar shifts in the pH optimum were obtained with NCS, the range of the shift being from pH 6·5 at 0·5 mM-NCS to pH 7·5 at 15 mM. There was a further slight shift to pH 7·3 when the NCS concentration was increased to 30 mM. The increase in substrate concentration

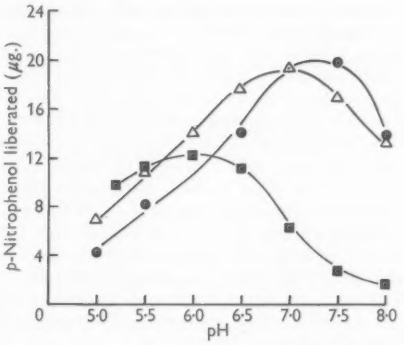


Fig. 1. Effect of potassium *p*-nitrophenyl sulphate concentration on the pH optimum of the arylsulphatase of the digestive juice of *Helix pomatia* acting in the presence of 0·25 M-acetic acid–sodium acetate buffer. ■, 0·5 mM; △, 6 mM; ●, 15 mM. The curves are quantitatively related to each other.

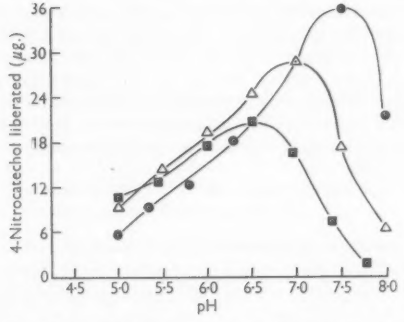


Fig. 2. Effect of dipotassium 2-hydroxy-5-nitrophenyl sulphate concentration on the pH optimum of the arylsulphatase of the digestive juice of *Helix pomatia* acting in the presence of 0·25 M-acetic acid–sodium acetate buffer. ■, 0·5 mM, △, 1·5 mM; ●, 15 mM. The curves are quantitatively related to each other.

Table 1. *Enzyme activities and Michaelis constants of the arylsulphatase of the digestive juice of* Helix pomatia *acting on potassium* p*-nitrophenyl sulphate and dipotassium* 2*-hydroxy-*5*-nitrophenyl sulphate at the lower and upper limits of the* pH *shift*

Arylsulphatase activity is expressed in terms of μg. of *p*-nitrophenol or 4-nitrocatechol liberated/ml. of digestive juice in 30 min. at 38° in the presence of 0·25 M-acetic acid–sodium acetate buffer.

Substrate	Substrate concn. (mM)	pH	Arylsulphatase activity	$10^4 K_m$
NPS	0·5	6·0	420 000	1·9
	15·0	7·3	700 000	17·9
NCS	0·5	6·5	620 000	0·3
	15·0	7·5	1060 000	12·5

which occurred with increasing pH was similar to that obtained with NPS.

Table 1 shows the K_m values (calculated from the plot of v against $v/[S]$) for NPS and NCS at the upper and lower limits of the pH shift. The table also records the activities of the juice at pH 6·0 and 0·5 mM-NPS, pH 7·3 and 15 mM-NPS, pH 6·5 and 0·5 mM-NCS and pH 7·5 and 15 mM-NCS. The ratio of the activities towards NPS under the two experimental conditions was of the order of 1:1·7; the corresponding ratio for NCS was also 1:1·7. This ratio of activities will be referred to hereafter as the ratio of activities at low and high pH.

NCS was used as substrate in subsequent experiments, because of its appreciable buffering power at high pH (cf. Roy, 1953).

Effect of time of incubation and enzyme concentration. The possibility existed that the shift in pH with substrate concentration might arise from an anomalous behaviour of the enzyme with the time of incubation similar to that observed for arylsulphatase A of human liver (Baum, Dodgson & Spencer, 1958; Baum & Dodgson, 1958). However, the time–activity curves at both low and high pH showed no anomalies although there was a steady decline in enzyme activity with time. Enzyme concentration–activity curves at low and high pH values were virtually linear and gave no indication of the presence of an enzyme with anomalous kinetics similar to those of human arylsulphatase A.

Attempted demonstration of the presence of two arylsulphatases

Inhibition experiments. The shift in pH optimum described earlier would be expected if the digestive juice contained two arylsulphatases, one having a high affinity for NPS and NCS and an optimum pH in the region of 6·0–6·5 and the other having a lower affinity for these substrates and an optimum pH in the region of 7·3–7·5. A similar multiplicity of arylsulphatases is known to occur in other organisms; for example, three distinct arylsulphatases occur in human and ox liver (Dodgson, Spencer & Thomas, 1955; Roy, 1956). An attempt was made to test this possibility by examining the effect of pH on *Helix* arylsulphatase in the presence of 25 mM-CN^- ions (an inhibitor of the type I enzymes) and 25 mM-PO_4^{3-} ions (inhibitory towards type II enzymes). An experiment of this type was used by Roy (1956) to distinguish between the types I and II arylsulphatases of ox liver. In the present work a substrate (NCS) concentration of 1·5 mM was selected, this being intermediate between the optimum concentrations obtained at pH 6·5 and 7·5 respectively. Fig. 3 shows that the pH optimum was not affected by cyanide or phosphate although the latter caused appreciable inhibition of enzyme activity. The curves provide no evidence for the co-existence of types I and II arylsulphatases in the digestive juice.

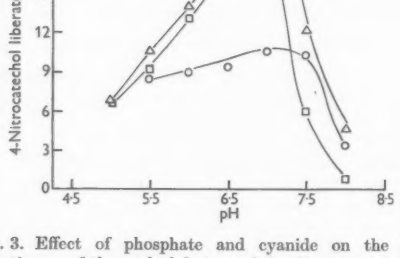


Fig. 3. Effect of phosphate and cyanide on the pH optimum of the arylsulphatase of the digestive juice of *Helix pomatia* acting on 1·5 mM-dipotassium 2-hydroxy-5-nitrophenyl sulphate in the presence of 0·25 M-acetic acid–sodium acetate buffer. △, Control curve; □, 25 mM-NaCN; ○, 25 mM-Na_2HPO_4–NaH_2PO_4.

Enzyme-fractionation experiments. The experiment described in the previous section did not eliminate the possibility that two arylsulphatases of the same type were present in the digestive juice (cf. the type II arylsulphatases A and B of human liver; Dodgson, Spencer & Wynn, 1956). Attempts were therefore made to separate two such enzymes by fractionation of the digestive juice with acetone and $(NH_4)_2SO_4$.

Two series of acetone fractionations were carried out, one at pH 7·0 when the juice (diluted 30-fold with water) was fractionated at 0° between the limits 0–45 and 45–70% (v/v) of acetone, and the other at pH 5·0 when seven successive fractions covering the range 0·80% (v/v) of acetone were obtained. The ratio of activities at low and high pH of each of the fractions was not significantly different from that of the unfractionated juice (Table 2). No fraction showed a reversal of the ratio such as might have been expected had separation of two arylsulphatases been achieved. pH–Activity curves of the 0–35 and 50–60% acetone fractions at NCS concentrations of 0·5 mM and 15 mM were virtually identical with those obtained with the unfractionated juice.

Similar fractionation experiments were carried out at 0° with $(NH_4)_2SO_4$. In one experiment a tenfold dilution of the juice was fractionated between the limits 0–45 and 45–70% saturation at pH 6·5, and in another a similar dilution of juice in 0·5 M-acetic acid–sodium acetate buffer was fractionated between the limits 0–40, 40–60 and 60–75% saturation at pH 5·5. Each fraction was dialysed for 18 hr. before assay at low and high pH

Table 2. *Distribution of arylsulphtase activity towards dipotassium 2-hydroxy-5-nitrophenyl sulphate after fractionation of the digestive juice of* Helix pomatia *with acetone or ammonium sulphate*

Experimental conditions for determination of the ratios of enzyme activity at low and high pH are given in the text.

Method of fractionation	pH of fractionation	Concn. of acetone (%, v/v) or $(NH_4)_2SO_4$ (% saturation)	Activity of fractions (% of total initial activity)	Ratio of enzyme activity at low and high pH
Acetone	7·0	0–45	6·5	1·0:1·7
		45–70	72·0	1·0:1·5
	5·0	0–35	1·3	1·0:1·8
		35–40	0·8	1·0:1·7
		40–45	1·3	1·0:1·8
		45–50	1·6	1·0:1·9
		50–60	45·0	1·0:1·7
		60–70	22·6	1·0:1·7
		70–80	0·4	1·0:1·4
$(NH_4)_2SO_4$	6·5	0–45	—	1·0:1·4
		45–70	—	1·0:1·5
	5·5	0–40	4·0	1·0:1·5
		40–60	42·0	1·0:1·7
		60–75	21·0	1·0:1·8

as before. The ratios of activities for these fractions (Table 2) were again very similar.

Electrophoresis experiments. Attempts were made to separate two enzymes by electrophoresis (horizontal) on Whatman no. 100 or 3MM paper in the presence of 0·1M-acetic acid–sodium acetate buffer at pH varying from 4·1 to 7·5. Electrophoresis was for 18 hr. at an ambient temperature of 4° and a potential gradient of 7v/cm. Strips (1 cm.) of the paper were subsequently incubated with NCS at low and high pH and enzyme activity was located by visual assessment of the 4-nitrocatechol colour after the addition of N-NaOH. Although appreciable movement of the enzyme (towards the cathode) occurred at below pH 6·0 separation of two enzymes was not achieved. At pH greater than 7·0 considerable loss of arylsulphatase activity was experienced. This appeared to be due to destruction of enzyme rather than removal of some cofactor or activator since the loss in activity was not restored when eluates of the 1 cm. strips were combined. Addition of boiled digestive juice or Mg^{2+} and Mn^{2+} ions to the pooled eluates did not restore enzyme activity.

Attempted removal of carbohydrate material from the juice. It will be observed from Table 2 that some enzyme activity appeared in all the fractions during the fractionation experiments. It seemed possible that the high concentration of polysaccharide material in the digestive juice was interfering with the fractionation procedures. Attempts were made to remove this polysaccharide material by the use at 0° of the $CHCl_3$ treatment of Sevag (1934). Although much polysaccharide was eliminated in this way, appreciable amounts still remained and the procedure was discontinued in view of the high losses of enzyme activity which were experienced. However, during the course of these experiments no significant change in the ratio of activities at low and high pH was observed.

In view of the problems posed by the presence of such large amounts of polysaccharide material it was decided to abandon the digestive juice as a source of arylsulphatase and investigate the possibility of using extracts of the digestive gland for further experiments.

Experiments with digestive-gland extracts

Preliminary work showed that the digestive gland was, as expected, a rich source of arylsulphatase, the enzyme being readily extracted from the gland with comparatively little associated carbohydrate material.

Enzyme extracts. The digestive glands (a total of 45 g. from 24 snails) were washed with ice-cold water and stored in the frozen state overnight. After thawing, the glands were suspended in 150 ml. of ice-cold water with the aid of a glass homogenizer (Potter & Elvehjem, 1936). The suspension was then incubated at 38° for 20 min. before centrifuging for 30 min. at 0° and 78 000 *g* av. (head no. 30) in the Spinco preparative ultracentrifuge. Aggregated fatty material was removed from the surface of the supernatant and the latter was then re-centrifuged at 0° and 105 000 *g* av. for 60 min. The clear supernatant was divided into two equal portions, one of which was dialysed at 2° against several changes of water for 40 hr. whereas the other was dialysed at 2° for 16 hr. against 60 vol. of 0·1M-disodium ethylenediaminetetra-acetate (EDTA) which had been adjusted to

pH 8·0 with ethanolamine. This portion was subsequently dialysed for 40 hr. at 2° against several changes of water. Each portion was adjusted to the same volume with water and stored in the frozen state. The preparations had to be diluted approximately 100 times for the enzyme experiments.

Effect of pH *and substrate concentration.* The behaviour of the arylsulphatase of the digestive gland towards variation of pH and substrate concentration in the presence of acetic acid–sodium acetate buffer was virtually identical with that shown by the digestive-juice enzyme. In the presence of 0·25 M-acetic acid–tris buffer there was greater enzyme activity (approx. 100% increase) and the pH shift varied from pH 6·6 at 0·5 mM-NCS to pH 7·4 at 15 mM-NCS. These experimental conditions were subsequently used to measure the ratio of activities at low and high pH respectively (cf. experiments on the digestive juice), the ratio for the extract being 1:1·5. K_m values obtained in the presence of 0·25 M-acetic acid–tris buffer at pH 6·6 and 7·4 (0·14 mM and 0·17 mM respectively) were appreciably different from those obtained at pH 6·5 and 7·5 respectively in 0·25 M-acetic acid–sodium acetate buffer (cf. Table 1). The enzyme activity of a typical preparation was such that in 15 min. at 38° approximately 3 mg. of 4-nitrocatechol was liberated/mg. of protein at low pH whereas the corresponding figure for high pH was 4·5 mg. No significant differences were observed between the EDTA-treated and the untreated preparation.

Effect of buffer concentration. Enzyme activity increased appreciably with increasing concentration of either acetic acid–sodium acetate or acetic acid–tris buffer (Fig. 4). In each case similar increases were obtained at both low and high pH. Subsequently acetic acid–tris buffer was used in most enzyme experiments because of its superior buffering power at high pH.

Effect of time of incubation and enzyme concentration. The effect of the time of incubation on enzyme activity towards NCS is shown in Fig. 5. There was a considerable decline in activity as incubation proceeded, particularly at the high pH. The enzyme could be pre-incubated for 60 min. before the addition of substrate, with only slight loss in activity. Linear enzyme concentration–activity curves were obtained at both low and high pH (Fig. 5).

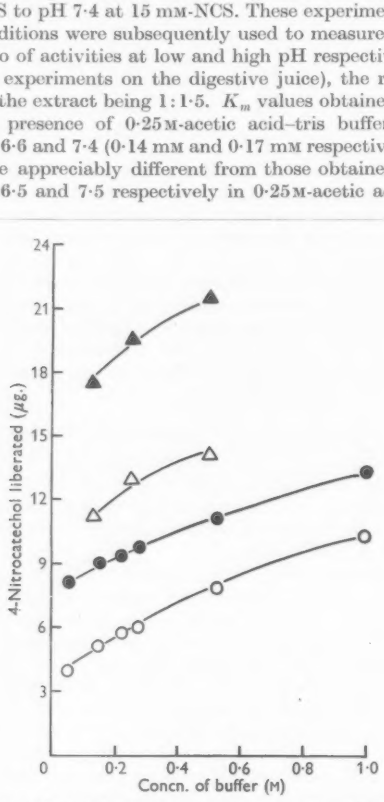


Fig. 4. Effect of buffer concentration on the arylsulphatase activity of a fixed amount of an extract of *Helix pomatia* digestive gland acting on dipotassium 2-hydroxy-5-nitrophenyl sulphate (NCS). ○, Acetic acid–sodium acetate buffer at 0·5 mM-NCS, pH 6·6; ●, acetic acid–sodium acetate buffer at 15 mM-NCS, pH 7·4; △, acetic acid–tris buffer at 0·5 mM-NCS, pH 6·6; ▲, acetic acid–tris buffer at 15 mM-NCS, pH 7·4.

DISCUSSION

Although many molluscs are known to be particularly rich in arylsulphatase (Dodgson & Spencer, 1953; Soda, 1936), *Helix pomatia* is undoubtedly the most potent source of the enzyme which has yet been described. The activity of the digestive juice which was observed during the present investigation is considerably higher than that quoted by Jarrige & Henry (1952). However, direct comparison of activities is not possible since the experimental conditions used by these workers for

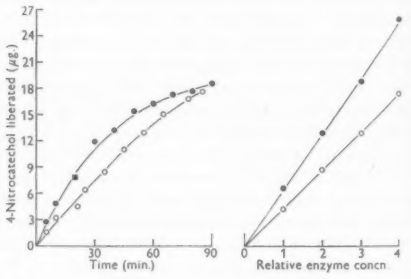


Fig. 5. Effect of time of incubation and enzyme concentration on the arylsulphatase of an extract of *Helix pomatia* digestive gland acting on dipotassium 2-hydroxy-5-nitrophenyl sulphate (NCS) in the presence of 0·25 M-acetic acid–tris buffer. ○, 0·5 mM-NCS, pH 6·6; ●, 15 mM-NCS, pH 7·4.

measuring activity (pH 5·2 and 0·5 mM-NPS in 0·1 M-acetic acid–sodium acetate buffer) were different from those used in the present work.

The significance of the high arylsulphatase activity of *Helix* is quite obscure, although it is possible that the enzyme might participate in the transfer of sulphate from arylsulphates to polysaccharides in a way similar to that recently suggested (Suzuki, Takahashi & Egami, 1959) for the arylsulphatase of the marine mollusc, *Charonia lampas*. Certainly *Helix* digestive juice contains appreciable amounts of sulphated polysaccharide material (Levene, 1925).

The shift in the optimum pH of *Helix* arylsulphatase which occurs with increasing substrate concentration is of interest. Similar shifts have been observed with other hydrolytic enzymes, for example, alkaline phosphatase (Morton, 1957) and β-glycosidase (Hofstee, 1955). No shifts of this type have previously been observed with other arylsulphatases although it must be stressed that in two cases only, namely the enzymes of *Patella vulgata* (Dodgson & Powell, unpublished results) and *Proteus vulgaris* (Dodgson, 1959), has any direct search for such shifts been made. It has already been mentioned that the co-existence of two distinct arylsulphatases in *Helix* could be responsible for the observed effects. The results described in this paper suggest that a single enzyme is responsible for the shifts; nevertheless direct proof of this was not obtained. If it is assumed that the effects can be attributed to a single enzyme then several possible explanations of the phenomenon can be advanced. The studies described in the next paper provide further evidence that a single enzyme is involved and eliminate a number of these possible explanations.

SUMMARY

1. A study has been made of the properties of a highly active arylsulphatase which is present in the digestive juice and in extracts of the digestive gland of the edible snail, *Helix pomatia*.

2. Within limits, the pH optimum of the enzyme shifts in the direction of higher pH as the concentration of substrate (either potassium *p*-nitrophenyl sulphate or dipotassium 2-hydroxy-5-nitrophenyl sulphate) is increased.

3. Fractionation of the digestive juice under varying conditions with acetone or ammonium sulphate together with paper-electrophoresis experiments suggest that the pH shift cannot be attributed to the presence of more than one arylsulphatase in the enzyme preparation.

We wish to thank Dr R. Henry for a generous gift of snail digestive juice. The work was aided by apparatus grants from The Wellcome Trust, The Royal Society and Imperial Chemical Industries Ltd. One of us (G.M.P.) is grateful to the Medical Research Council for a studentship.

REFERENCES

Baum, H. & Dodgson, K. S. (1958). *Biochem. J.* **69**, 573.
Baum, H., Dodgson, K. S. & Spencer, B. (1958). *Biochem. J.* **69**, 567.
Burkhardt, G. N. & Lapworth, A. (1926). *J. chem. Soc.* p. 684.
Dastugue, G., Bastide, P. & Tronche, J. (1957). *9th Congrès Soc. Pharm. France, Clermont, Juin.*
Dodgson, K. S. (1959). *Enzymologia*, **20**, 301.
Dodgson, K. S. & Spencer, B. (1953). *Biochem. J.* **55**, 315.
Dodgson, K. S. & Spencer, B. (1956). *Biochim. biophys. Acta*, **21**, 175.
Dodgson, K. S. & Spencer, B. (1957). *Meth. biochem. Anal.* **4**, 211.
Dodgson, K. S., Spencer, B. & Thoms, J. (1955). *Biochem. J.* **59**, 29.
Dodgson, K. S., Spencer, B. & Wynn, C. H. (1956). *Biochem. J.* **62**, 500.
Hofstee, B. H. J. (1955). *Arch. Biochem. Biophys.* **59**, 398.
Jarrige, P. & Henry, R. (1952). *Bull. Soc. Chim. biol., Paris*, **34**, 872.
Levene, P. A. (1925). *J. biol. Chem.* **65**, 683.
Morton, R. K. (1957). *Biochem. J.* **65**, 674.
Potter, V. R. & Elvehjem, C. G. (1936). *J. biol. Chem.* **114**, 495.
Roy, A. B. (1953). *Biochem. J.* **53**, 12.
Roy, A. B. (1956). *Biochem. J.* **64**, 651.
Sevag, M. G. (1934). *Biochem. Z.* **273**, 419.
Soda, T. (1936). *J. Fac. Sci. Tokyo Univ.*, **3**, 149.
Spencer, B. (1959). *Biochem. J.* **73**, 442.
Suzuki, S., Takahashi, N. & Egami, F. (1959). *J. Biochem., Tokyo*, **46**, 1.

Studies on Sulphatases

27. THE PARTIAL PURIFICATION AND PROPERTIES OF THE ARYLSULPHATASE OF THE DIGESTIVE GLAND OF *HELIX POMATIA*

By K. S. DODGSON and GILLIAN M. POWELL
Department of Biochemistry, University of Wales, Newport Road, Cardiff

(*Received* 24 *April* 1959)

In the preceding paper (Dodgson & Powell, 1959) it was shown that an arylsulphatase present in the digestive juice and the digestive gland of the edible snail exhibited a shift in optimum pH as the concentration of substrate was increased. This paper describes the partial purification of the digestive-gland enzyme and provides further evidence that the shift in optimum pH is not due to the presence of more than one arylsulphatase.

MATERIALS AND METHODS

Substrates. Potassium *p*-nitrophenyl sulphate (NPS) and dipotassium 2-hydroxy-5-nitrophenyl sulphate (NCS) were prepared as described by Dodgson & Powell (1959); the monopotassium salts of 4-hydroxy-2-nitrophenyl sulphate and 4-hydroxy-3-nitrophenyl sulphate were prepared by the method of Smith (1951).

Determination of enzyme activity. Arylsulphatase activity towards NPS and NCS was measured by the methods previously described (Dodgson & Powell, 1959). When the monopotassium salts of 4-hydroxy-3-nitrophenyl sulphate and 4-hydroxy-2-nitrophenyl sulphate were used as substrates, the method of determining enzyme activity was identical with that used for NPS and NCS, except that the spectrophotometric measurement of the final colour was at 540 mμ. Except where otherwise stated, incubation of enzyme (0·2 ml.) and substrate (0·2 ml.) was for 15 min. in the presence of 0·25 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris), which had been adjusted to the required pH with acetic acid. Enzyme concentrations were adjusted to give final spectrophotometric readings ($\log I_0/I$) of less than 1·0. For reasons which are outlined below, enzyme and substrate were always mixed by rotating the reaction tubes between the hands.

Determination of nucleic acid. The ratio of the spectrophotometric readings at 280 and 260 mμ was used to determine nucleic acid (Warburg & Christian, 1941).

Determination of protein. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Ratio of enzyme activity at low and high pH. Dodgson & Powell (1959) for convenience used the terms low and high pH to describe the two sets of experimental conditions for measuring enzyme activity. In the present work low pH conditions for NCS were pH 6·6 and 0·5 mM, and for NPS pH 6·25 and 0·5 mM. The corresponding high pH conditions were pH 7·4 and 15 mM-NCS and pH 7·5 and 15 mM-NPS.

EXPERIMENTAL AND RESULTS

Purification procedure

Stage 1. An aqueous extract of the digestive glands of starved snails was made as described by Dodgson & Powell (1959). The clear extract was dialysed for 24 hr. against water (100 vol.) at 2°.

Stage 2. The extract was adjusted to pH 6·0 and treated at 0° with M-$MnCl_2$ (5 ml. for each 50 ml. of extract) at the same pH. After standing at 0° for 45 min. the copious precipitate was separated by centrifuging at 0°. The clear supernatant was adjusted to pH 8·0 with N-NaOH and, after standing at 0° for 20 min., the precipitate was removed by centrifuging. The supernatant was then dialysed overnight at 2° against water (50 vol.).

Stage 3. Acetone was added slowly (with gentle stirring) to the enzyme solution at −5° to give a final concentration of acetone of 47% (v/v). After standing at −5° for 30 min. the precipitate was separated by centrifuging at −5° and discarded. The concentration of acetone in the supernatant was then increased to 57% (v/v) and, after standing at −5° for 15 min., the precipitate was collected by centrifuging at the same temperature. The precipitate was dissolved in ice-cold water (50 ml. for each 50 ml. of the original extract) and dialysed overnight at 2° against water (50 vol.).

Stage 4. The enzyme solution was adjusted to pH 5·0 with a trace of acetic acid, and solid $(NH_4)_2SO_4$ was added over a period of 30 min., with gentle stirring until the solution was 45% saturated with respect to $(NH_4)_2SO_4$. Frequent checks on the pH of the solution were made during this addition and when necessary it was readjusted to pH 5·0 with a trace of N-NaOH. After standing at 0° for 2 hr. the precipitate was removed by centrifuging and the concentration of $(NH_4)_2SO_4$ in the supernatant was increased to 70% saturation. The whole was kept at 0° for 2 hr., when the precipitate was separated by centrifuging, dissolved in ice-cold water (30 ml./50 ml. of original extract) and dialysed overnight at 2° against water (50 vol.).

Stage 5. The dialysate was adjusted to pH 7·0 and fractionated between 55 and 70% saturation with $(NH_4)_2SO_4$. After standing for 2 hr. at 0°, the precipitate was separated, dissolved in ice-cold water (30 ml./50 ml. of original extract) and dialysed for 24 hr. against several changes of water at 2°. The final enzyme solution was stored in the frozen state in stoppered plastic containers.

Table 1 shows the enzyme activities towards NCS at stages 1, 2, 3 and 5 of a typical purification. One of the main aims of the procedure was to remove nucleic acid, in view of its known tendency to combine readily with protein and thereby to be a possible contributory factor to the

Table 1. *Arylsulphatase activity of the digestive gland of* Helix pomatia *at various stages of the purification procedure*

Enzyme activity was measured against dipotassium 2-hydroxy-5-nitrophenyl sulphate (NCS) in the presence of 0·25 M-acetic acid–tris buffer at low pH (pH 6·6 and 0·5 mM-NCS) and high pH (pH 7·4 and 15 mM-NCS) as described in the text. One unit of arylsulphatase activity is equivalent to 1 μg. of 4-nitrocatechol liberated/15 min. at 38°.

Purification stage	Enzyme activity (units/mg. of protein)		Ratio of activities at low and high pH	Concn. of nucleic acid (%)
	Low pH	High pH		
1	2 980	4 430	1:1·5	2·5
2	3 800	5 500	1:1·4	0
3	21 000	29 400	1:1·4	—
5	54 850	76 500	1:1·4	—

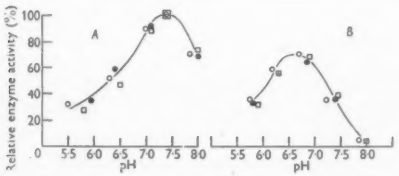


Fig. 1. Effect of pH on the arylsulphatase of *Helix pomatia* digestive gland at stages 1, 3 and 5 of the purification procedure. *A*, Measured against 15 mM-dipotassium 2-hydroxy-5-nitrophenyl sulphate (NCS) in the presence of 0·25 M-acetic acid–tris buffer; *B*, measured against 0·5 mM-NCS in the same buffer. ●, Stage 1; □, stage 3; ○, stage 5. For the purpose of clarity all the experimental points have been plotted as a percentage of the enzyme activity obtained at pH 7·4 and 15 mM-NCS.

Table 2. *Michaelis constants at* pH 6·6 *and* 7·4 *for the arylsulphatase of* Helix pomatia *digestive gland at stages* 1, 3 *and* 5 *of the purification procedure*

The enzyme was acting on dipotassium 2-hydroxy-5-nitrophenyl sulphate in the presence of 0·25 M-acetic acid–tris buffer at 38°.

Purification stage	$10^4 K_m$	
	pH 6·6	pH 7·4
1	1·4	1·7
3	1·3	4·7
5	0·9	7·3

shift in pH optimum. Treatment with $MnCl_2$ was effective in eliminating nucleic acid. On the other hand, attempted removal of nucleic acid with protamine (cf. Dodgson & Lloyd, 1957) resulted in substantial losses in enzyme activity.

Appreciable losses in enzyme activity occurred during purification, but these losses were considerably increased if the procedure was not strictly adhered to. The final preparation usually contained about 10% of the activity of the initial extract. A number of other procedures which were attempted, including the use of adsorbents, electrophoresis and fractionation on carboxymethylcellulose ion-exchange columns, resulted in large and often complete losses of enzyme activity.

Properties of the enzyme at various stages of purification

Table 1 shows that the ratio of enzyme activity at low and high pH did not vary significantly during purification. This finding provides further evidence for the existence of one arylsulphatase only in the enzyme preparation. In order to obtain additional support for this view the properties of the enzyme preparation at stages 1, 3 and 5 were compared.

Effect of pH *and substrate concentrations.* Fig. 1 shows the pH–activity curves for the three stages with NCS at low and high concentrations. The curves obtained for each stage were similar. Substrate concentration–activity curves towards NCS at pH 6·6 and 7·4 for the three stages provided no indication of the presence of more than one arylsulphatase. K_m values for each stage, calculated from the plot of v against $v/[S]$, are listed in Table 2. At high pH there was a steady decrease in the affinity of enzyme for substrate as the purity of the preparation increased, whereas, at low pH, the opposite effect was obtained.

Effect of time of incubation and enzyme concentration. Fig. 2 shows the effects of the time of incubation on the arylsulphatase activity of the three stages when measured at both low and high pH. In each case there was a marked decline in enzyme activity as the reaction proceeded. All three stages gave linear enzyme concentration–activity curves at both low and high pH. There was no evidence from the curves of the presence of an endogenous inhibitor or activator.

Effect of buffer concentration. The increase in enzyme activity with increasing concentration of buffer (either acetic acid–tris or acetic acid–sodium acetate) which had previously been observed for the crude extract of the digestive gland (Dodgson & Powell, 1959) was still obtained with stages 3 and 5. In each case the extent of the increase when measured at low pH paralleled that obtained at high pH, suggesting that the effect was due to a single enzyme.

Instability of the enzyme

During the initial studies on the purification of the digestive-gland enzyme considerable difficulty was experienced in obtaining satisfactory duplicate determinations when measuring enzyme activity. The reason for this was ultimately traced to the fact that enzyme and substrate solutions were being mixed by agitation of the centrifuge tubes in which the determinations were made. Losses in enzyme activity were occurring as a result of this procedure. These losses were eliminated when substrate and enzyme were mixed by rotation of the reaction tubes between the hands. A similar effect has been noted by Morton (1957) with alkaline phosphatase.

A series of experiments showed that when the stage 1 enzyme and the substrate (both at 38°) were mixed by brief agitation, as opposed to rotation, up to 10% loss in enzyme activity occurred. With the stage 5 enzyme the corresponding figure approximated to 20%. Agitation of the warm enzyme solution before the addition of substrate resulted in only slight losses in enzyme activity. Other experiments were made in which the incubation mixtures were briefly agitated every 1 min. during the incubation period (15 min.). With the stage 1 enzyme up to 30% of the activity was lost as a result of this treatment, whereas with the stage 5 enzyme between 60 and 70% of the activity disappeared. The losses in activity were not due to adsorption of the enzyme on glass surfaces [cf. acid phosphatase (Jeffree, 1957) and testicular hyaluronidase (Rasmussen, 1954)] since similar losses were obtained with plastic tubes.

Other observations indicated that the stage 5 enzyme was particularly unstable. Thus appreciable losses in enzyme activity occurred during any procedure where frothing of the enzyme solution occurred. Freezing and thawing also resulted in destruction of the enzyme, as did storage of the enzyme (particularly diluted enzyme) in the liquid state. It is perhaps significant that, in all cases where loss of enzyme activity was experienced, the percentage decrease in activity as measured at low pH approximately paralleled that measured at high pH.

Further observations on the pH *shift*

Action of the enzyme on other substrates. A pH shift similar to that observed for the stage 5 enzyme acting on NCS was also obtained with other arylsulphatase substrates. With NPS in the presence of 0·25 M-acetic acid–tris buffer the shift ranged from pH 6·25 at a substrate concentration of 0·5 mM to pH 7·5 at 15 mM. Table 3 records the relative enzyme activities and Michaelis constant (K_m, calc. from the plot of v against $v/[S]$) for NPS and NCS at the lower and upper limits of the pH shift. A comparison of the values for NCS and NPS at low pH together with a comparison of the corresponding values at high pH shows that the enzyme exhibits relatively greater affinity and activity towards NCS.

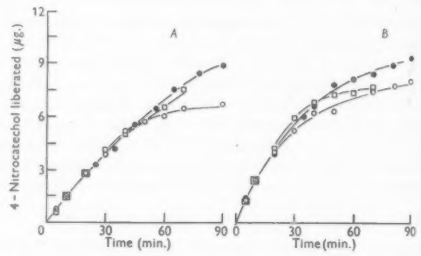


Fig. 2. Effect of time of incubation on the arylsulphatase of *Helix pomatia* digestive gland at stages 1, 3 and 5 of the purification procedure. *A*, Measured against 0·5 mM-dipotassium 2-hydroxy-5-nitrophenyl sulphate (NCS) in the presence of 0·25 M-acetic acid–tris buffer, pH 6·6; *B*, measured against 15 mM-NCS in the same buffer, pH 7·4. ●, Stage 1; □, stage 3; ○, stage 5. Stage 3 and 5 enzyme concentrations were adjusted so that the amount of 4-nitrocatechol liberated in 10 min. was equal to that liberated by the stage 1 enzyme in the same period.

Table 3. *Relative enzyme activities and Michaelis constants (K_m) for the arylsulphatase of* Helix pomatia *digestive gland at stage 5 of the purification procedure*

The enzyme was acting on dipotassium 2-hydroxy-5-nitrophenyl sulphate (NCS) and potassium *p*-nitrophenyl sulphate (NPS) at the lower and upper limits of the pH shift in the presence of 0·25 M-acetic acid–tris buffer. Arylsulphatase activity is expressed in terms of μg. of 4-nitrocatechol or *p*-nitrophenol liberated/mg. of protein/15 min. at 38°.

Substrate	Concn. of substrate (mM)	pH	Arylsulphatase activity	$10^4 K_m$
NPS	0·5	6·25	18 800	2·7
	15·0	7·5	49 700	51·0
NCS	0·5	6·6	54 850	0·9
	15·0	7·4	76 500	7·3

Fig. 3 shows the effect of increasing substrate concentration on the optimum pH of the stage 5 enzyme acting on the monopotassium salts of 4-hydroxy-2-nitrophenyl sulphate and 4-hydroxy-3-nitrophenyl sulphate in the presence of 0·25 M-acetic acid–sodium acetate buffer. In each case there was a shift in pH optimum but the extent of the shift was less marked than that obtained with NPS or NCS.

Treatment with disodium ethylenediaminetetra-acetate. Mention has already been made (Dodgson & Powell, 1959) that treatment of the crude enzyme extract of the digestive gland with sodium ethylenediaminetetra-acetate (EDTA) had no effect on the shift in pH optimum. This suggested that the presence of a metal activator or inhibitor could not be responsible for the phenomenon (cf. alkaline phosphatase, Morton, 1957). Further attempts were made to remove possible trace metals from the stage 5 enzyme with a procedure similar to that described by Morton (1957).

The enzyme solution (1 ml.) was mixed with 4 ml. of 0·2 M-EDTA which had been adjusted to pH 8·0 with ethanolamine. The solution was then dialysed for 24 hr. at at 2° against 1 l. of 0·1 M-EDTA which had been adjusted to the same pH, and subsequently for 48 hr. against two changes of water (2 l. in all). The activity of the final dialysate at low and high pH was compared with that of the original enzyme preparation and with enzyme which had been subjected to the dialysis procedures except that water was substituted for EDTA. As a further control, 1 ml. of enzyme was diluted with 4 ml. of water and allowed to stand at 2° for the same period of time. All enzyme solutions were adjusted to the same volume before assay.

No loss in enzyme activity occurred as a result of the treatment with EDTA. On the other hand, some loss in enzyme activity was observed with the enzyme preparation which had been dialysed against water only (17 % loss) and with the preparation which had been allowed to stand at 2° (6 % loss). The presence of an endogenous metal activator or inhibitor in the stage 5 enzyme therefore seems unlikely. Mg^{2+}, Mn^{2+} and Zn^{2+} ions had no activating effect on the enzyme (see Table 4).

Possible inhibitory effects of hydroxyl ions. The shift in pH optimum could possibly be explained if OH^- ions were acting as competitive inhibitors of the enzyme. Such inhibition would be greatest at low substrate concentrations and would result in an apparent shift in the pH optimum of the enzyme in the direction of lower pH as the substrate concentration was decreased. To explore this possibility a study was made of the effect of substrate (NCS) concentration on the activity of three different concentrations of the enzyme at pH 7·4 in the presence of 0·25 M-acetic acid–tris buffer. Under these circumstances the concentration of OH^- ions remained constant but the concentration of the enzyme and hence the enzyme–inhibitor (OH^- ions) ratio was varied. Fig. 4 shows the substrate concentration–activity curves obtained. K_m values (calc. from the plot of v against $v/[S]$) for relative enzyme concentrations of 1, 2 and 3 were 0·52, 0·73 and 0·64 mM respectively. These values can probably be considered as identical within the limits of experimental error and provide no indication of inhibition by OH^- ions.

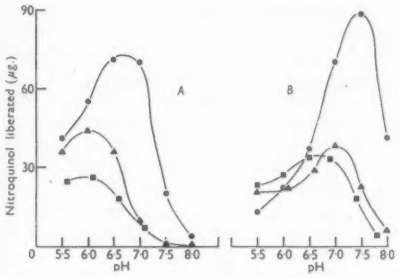


Fig. 3. Effect of substrate concentration on the pH optimum of the arylsulphatase of *Helix pomatia* digestive gland (stage 5) acting on: *A*, monopotassium 4-hydroxy-3-nitrophenyl sulphate in the presence of 0·25 M-acetic acid–sodium acetate buffer; *B*, monopotassium 4-hydroxy-2-nitrophenyl sulphate in the same buffer. ■, 0·5 mM; ▲, 15 mM; ●, 15 mM. The concentration of enzyme used to obtain the curves in *A* was five times that used to obtain the curves in *B*.

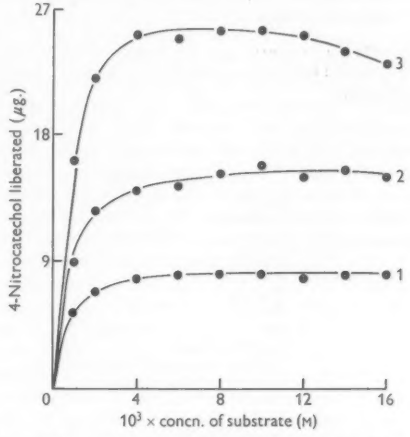


Fig. 4. Effect of the concentration of dipotassium 2-hydroxy-5-nitrophenyl sulphate on three different concentrations of the stage 5 enzyme of *Helix pomatia* digestive gland acting in the presence of 0·25 M-acetic acid–tris buffer, pH 7·4. The curves are numbered in terms of the relative enzyme concentration used.

Effect of certain compounds on enzyme activity at low and high pH. A study was made of the effects of possible inhibitors and activators on enzyme activity towards NPS and NCS at both low and high pH. In these experiments a slight modification of the method of measuring enzyme activity was made since some of the compounds tested (e.g. Na_2SO_3, $NaHSO_3$, phenylhydrazine and hydroxylamine) are capable, under certain conditions, of hydrolysing one or both of the assay substrates (Dodgson & Powell, unpublished results).

Enzyme (0·2 ml.) and inhibitor (0·2 ml. of a 0·1 M-solution in 0·5 M-acetic acid–tris buffer at the appropriate pH) were pre-incubated for 3 min. at 38° before adding 0·4 ml. of a solution of the substrate in 0·25 M-acetic acid–tris buffer at the same pH. Suitable controls were made in which 0·5 M-acetic acid–tris buffer was substituted for buffered inhibitor and, as a control of possible non-enzymic hydrolysis of substrate by inhibitor, further determinations were made in which inhibitor and boiled enzyme were incubated with substrate.

Table 4 shows that phosphate, sulphite, bisulphite and fluoride were the most powerful inhibitors of *Helix* arylsulphatase. In contrast, cyanide had little effect on enzyme activity. The results provide no clear indication whether a single enzyme only is involved, although, in general, where inhibition occurs the effects are seen at both low and high pH. It will be observed that phosphate, fluoride, sulphite and sulphate exert greatest inhibitory effects at low pH values. On the other hand, semicarbazide, hydroxylamine and (towards NCS) phenylhydrazine exert their greatest effects at high pH values. This may at first suggest

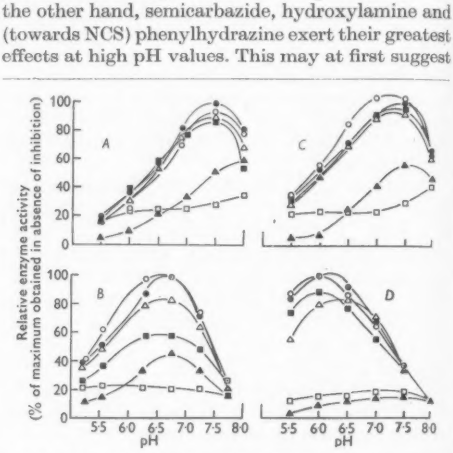


Fig. 5. Effect of certain compounds (at a concentration of 25 mM) on the pH optimum of the arylsulphatase of *Helix pomatia* digestive gland (stage 5) acting on dipotassium 2-hydroxy-5-nitrophenyl sulphate (NCS) and potassium *p*-nitrophenyl sulphate (NPS) in the presence of 0·25 M-acetic acid–tris buffer. *A*, 15 mM-NCS; *B*, 0·5 M-NCS; *C*, 15 mM-NPS; *D*, 0·5 mM-NPS. ●, Control; ○, NaCl; ■, NaCN; □, Na_2HPO_4–NaH_2PO_4; ▲, NaF; △, Na_2SO_4.

Table 4. *Effects of certain compounds on the activity of the arylsulphatase of the digestive gland of* Helix pomatia *acting on potassium* p*-nitrophenyl sulphate and dipotassium* 2*-hydroxy-5-nitrophenyl sulphate at low and high* pH

Results are expressed as a percentage of the enzyme activity obtained in the absence of inhibitor or activator. The terms low and high pH are explained in the text. Except where indicated, the final concentration of inhibitor or activator was 25 mM.

	Enzyme activity (%)			
	NPS		NCS	
Compound added	Low pH	High pH	Low pH	High pH
Na_2SO_3	4	5	1	5
$NaHSO_3$	1	6	1	6
NaF	35	47	11	55
Na_3PO_4	20	24	18	26
$Na_4P_2O_7$	48	95	29	86
Na_2SO_4	61	86	89	95
NaCN	88	91	94	96
NaCl	100	94	100	105
Sodium citrate	92	80	94	87
Sodium benzene sulphonate	90	95	64	92
Potassium ethyl sulphate	100	86	86	102
Urea	100	100	97	95
Hydroxylamine	65	34	89	51
Semicarbazide	102	91	98	83
Hydrazine	101	103	100	89
Phenylhydrazine	77	61	67	83
$MgCl_2$*	83	85	90	89
$MnCl_2$*	80	81	89	84
$ZnCl_2$*	64	77	74	84

* Final concn. 1 mM.

that two enzymes are present in the enzyme preparation. However, it is more probable that the contrast merely reflects the difference between anionic inhibitors, on the one hand, and those which can be regarded as cationic, on the other.

Fig. 5 shows the effects on the pH curves at low and high concentrations of substrate (NCS and NPS) of some of the compounds which were tested as inhibitors. With the exception of fluoride and phosphate, which tended (particularly when measured at high substrate concentrations) to shift the pH optima in the direction of higher pH, the compounds had little effect on the pH optima. A similar effect of fluoride and phosphate had previously been noted for the type 2 arylsulphatase of *Proteus vulgaris* (Dodgson, 1959).

DISCUSSION

The collective results provide some indication that the shift in pH optimum with increasing substrate concentration cannot be attributed to the presence of more than one arylsulphatase in the enzyme preparation. Mention has already been made that a number of other hydrolytic enzymes exhibit a similar phenomenon (alkaline phosphatase, Morton, 1957; β-glucosidase, Hofstee, 1955; β-glucuronidase, Cox, 1959), and a number of possible explanations of this effect can be advanced. The theoretical background to these explanations has been dealt with by Bull (1954) and by Friedenwald & Maengwyn-Davies (1954) and will not therefore be reiterated here. It is sufficient to say that the effect might be observed with any enzyme–substrate interaction in which several equilibria are involved or where the reaction at the specific substrate-binding site of the enzyme, resulting in the formation of the enzyme–substrate complex, is independent of the subsequent reaction of the substrate with the catalytic site(s) of the enzyme. Situations in which the overall reaction involves a number of different equilibria could result, for example, from the participation of an activator (e.g. a metal, H^+ or OH^- ions) in the catalytic process. An explanation based on these lines has been advanced by Morton (1957) to account for the variation with substrate concentration of the optimum pH of alkaline phosphatase, an enzyme whose activity depends on the presence of a metal (usually Mg^{2+} ions). Morton has pointed out that, for the overall enzymic reaction, ten different equilibria must be considered. Three of these can be regarded as competitive routes for the formation of the metal–enzyme–substrate complex and a fourth shows that the effective concentration of metal activator will be influenced by the concentration of OH^- ions and hence pH. Motzok & Branion (1959), working with chick alkaline phosphatase, also suggest that the inhibitory effect of OH^- ions might explain the shift in pH optimum exhibited by this enzyme. However, similar considerations do not apply to *Helix* arylsulphatase since OH^- ions appear to have no inhibitory effect on the enzyme, the activity of which appears to be independent of the presence of a metal activator. Mention has previously been made that the enzyme concentration–activity curves for *Helix* arylsulphatase provide no indication of the presence of endogenous inhibitors or activators.

Another type of situation where several equilibria must be considered will arise when the substrate is capable of existing in more than one ionic form (Friedenwald & Maengwyn-Davies, 1954). Cox (1959) has suggested that the shift in pH optimum with change in concentration of substrate which occurs when molluscan β-glucuronidase is acting on phenolphthalein glucuronide can be explained in this way. Cox suggests that the enzymic reaction requires the participation of un-ionized substrate. At low substrate concentrations the concentration of un-ionized phenolphthalein glucuronide (pK of the substrate carboxyl group is about 3·0) might be the limiting factor and decrease in pH towards 3·0 would increase the un-ionized substrate concentration. It follows that the lower the substrate concentration, the lower would be the apparent optimum pH. In *Helix* arylsulphatase the free phenolic hydroxyl group present in one of the substrates used, namely NCS, is known to have pK about 6·5 (Dodgson, Spencer & Williams, 1955). Although no direct check has been made similar considerations will probably apply to the free phenolic hydroxyl groups of two of the other substrates used, namely potassium 4-hydroxy-3-nitrophenyl sulphate and potassium 4-hydroxy-2-nitrophenyl sulphate. With all three substrates, over the pH range studied, the proportions of ionized (with respect to the phenolic hydroxyl group) to un-ionized substrate will vary with pH. However, this apparently in no way contributes to the observed shift in pH optimum since the shift is also obtained with NPS, a substrate which does not possess a free phenolic hydroxyl group.

Hofstee (1955) has shown that the pH optimum of β-glucosidase shifts from about 3·5 at concentrations of substrate (salicylic acid β-glucoside) below 1 mM to about 4·5 at a substrate concentration of 33 mM. Hofstee attributes this shift to the difference in the influence of pH on K_m and $V_{max.}$, i.e. on the formation and the breakdown of the enzyme–substrate complex respectively. This may be the explanation of the pH shift with *Helix* arylsulphatase, although there is, as yet, no evidence to support this possibility. However with human arylsulphatase B (Dodgson & Wynn, 1958) and ox arylsulphatase B (Webb & Morrow, 1959),

both of which exhibit a number of properties in common with the *Helix* enzyme, the formation of the enzyme–substrate complex and the subsequent breakdown thereof can be influenced independently by variation of experimental conditions. No attempt has yet been made to test this possibility for the *Helix* enzyme. Buffer concentration has a marked effect on the activity of this enzyme and this effect probably reflects the comparative impurity of the enzyme preparation. Thus a similar effect has been observed by Dodgson & Wynn (1958) for comparatively crude preparations of both human and ox arylsulphatase B, but the effect is no longer obtained with the highly purified enzymes (Dodgson & Wynn, 1958; Webb & Morrow, 1959). It therefore seems desirable to obtain *Helix* arylsulphatase in a highly purified form before pursuing further the problem of the shift in pH optimum. As a preliminary to this further purification a study is now being made of possible ways of protecting the enzyme from denaturation during the purification procedure.

Dodgson & Spencer (1957) have suggested that the arylsulphatase enzymes appear to fall into two groups. The type I arylsulphatases have a high affinity for, and are particularly active towards, simple arylsulphates such as NPS. They are not markedly affected by phosphate and fluoride but are strongly inhibited by cyanide. In contrast the type II arylsulphatases show greatest affinity and activity towards more complex arylsulphates such as NCS and are strongly inhibited by phosphate and fluoride but not by cyanide. On the basis of these suggestions *Helix* arylsulphatase would be classified as a type II enzyme. Thus the enzyme shows a greater affinity and activity towards NCS than towards NPS (Table 3) and, although largely unaffected by cyanide, is inhibited strongly by phosphate and fluoride (Table 4). However, in certain respects the enzyme is unlike any other type II arylsulphatase previously studied. Thus the pH shift which occurs with increasing substrate concentration does not occur with the two other type II arylsulphatases (from *Patella vulgata* and *Proteus vulgaris*) which have so far been investigated (Dodgson & Powell, unpublished results; Dodgson, 1959). Moreover, at least three of the type II arylsulphatases (human and ox arylsulphatases B and the enzyme of *Proteus vulgaris*) are known to be activated strongly (several-fold) by chloride when acting on NPS (Dodgson & Wynn, unpublished results; Webb & Morrow, 1959; Dodgson, 1959). This effect is not shown by the *Helix* enzyme (Fig. 5). Further work is continuing in these Laboratories to establish whether the arylsulphatases can be justifiably classified into two distinct types or whether there exists a whole 'spectrum' of enzymes between the two extremes.

Finally, with β-glucosidase, alkaline phosphatase and β-glucuronidase, all of which exhibit shifts in pH optima with increase in substrate concentration, the enzymes are able to catalyse the transfer of glucose, phosphate and glucuronic acid respectively to acceptors other than water (see, for example, Rabaté, 1935; Morton, 1958; Fishman & Green, 1957). The possibility that *Helix* arylsulphatase is capable of transferring sulphate from arylsulphates to acceptors other than water is now being examined.

SUMMARY

1. The arylsulphatase of the digestive gland of *Helix pomatia* has been partially purified by treatment with manganese chloride followed by fractionation with acetone and ammonium sulphate.

2. The enzyme became progressively more unstable as the purity increased but no other significant changes in the properties of the enzyme occurred during the purification procedure, and the final preparation was still exhibiting a shift in pH optimum in the direction of higher pH as the substrate concentration was increased.

3. Evidence suggests that the observed shift in pH optimum cannot be attributed to the presence of more than one arylsulphatase in the enzyme preparation.

4. Some arguments which have been used to explain similar shifts in pH optima which occur with other hydrolytic enzymes (e.g. alkaline phosphatase) have been shown to be inapplicable to the *Helix* enzyme.

5. The relative behaviour of the enzyme towards potassium *p*-nitrophenyl sulphate and dipotassium 2-hydroxy-5-nitrophenyl sulphate together with the effects of certain inhibitors suggests that the enzyme can be classified as a type II arylsulphatase.

The work was aided by apparatus grants from The Wellcome Trust, The Royal Society and Imperial Chemical Industries Ltd. One of us (G.M.P.) is grateful to the Medical Research Council for a studentship.

REFERENCES

Bull, H. B. (1954). In *The Mechanism of Enzyme Action*. Ed. by McElroy, W. D. & Glass, B. Baltimore: The Johns Hopkins Press.

Cox, R. I. (1959). *Biochem. J.* **71**, 763.

Dodgson, K. S. (1959). *Enzymologia*, **20**, 301.

Dodgson, K. S. & Lloyd, A. G. (1957). *Biochem. J.* **66**, 532.

Dodgson, K. S. & Powell, G. M. (1959). *Biochem. J.* **73**, 666.

Dodgson, K. S. & Spencer, B. (1957). *Meth. biochem. Anal.* **4**, 211.

Dodgson, K. S., Spencer, B. & Williams, K. (1955). *Biochem. J.* **61**, 374.

Dodgson, K. S. & Wynn, C. H. (1958). *Biochem. J.* **68**, 387.
Fishman, W. H. & Green, S. (1957). *J. biol. Chem.* **225**, 435.
Friedenwald, J. S. & Maengwyn-Davies, G. D. (1954). In *The Mechanism of Enzyme Action*. Ed. by McElroy, W. D. & Glass, B. Baltimore: The Johns Hopkins Press.
Hofstee, B. H. J. (1955). *Arch. Biochem. Biophys.* **59**, 398.
Jeffree, G. M. (1957). *Biochim. biophys. Acta*, **23**, 155.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Morton, R. K. (1957). *Biochem. J.* **65**, 674.
Morton, R. K. (1958). *Biochem. J.* **70**, 139.
Motzok, I. & Branion, H. D. (1959). *Biochem. J.* **72**, 177.
Rabaté, M. J. (1935). *Bull. Soc. Chim. biol., Paris*, **17**, 572.
Rasmussen, P. S. (1954). *Acta chem. scand.* **8**, 633.
Smith, J. N. (1951). *J. chem. Soc.* p. 2861.
Warburg, O. & Christian, W. (1941). *Biochem. Z.* **310**, 384.
Webb, E. C. & Morrow, P. F. W. (1959). *Biochem. J.* **73**, 7.

The Biosynthesis of Porphyrins from Porphobilinogen by *Rhodopseudomonas spheroides*

2. THE PARTIAL PURIFICATION AND SOME PROPERTIES OF PORPHOBILINOGEN DEAMINASE AND UROPORPHYRINOGEN DECARBOXYLASE*

By D. S. HOARE† and H. HEATH
Department of Chemical Pathology, University College Hospital Medical School, London, W.C. 1

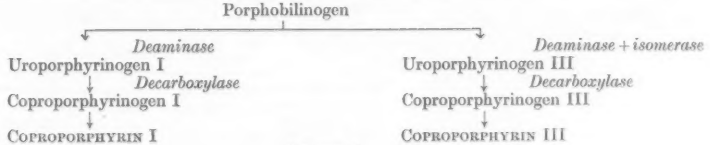
(*Received* 26 *May* 1959)

It has been already established that preparations from the photosynthetic bacterium *Rhodopseudomonas spheroides* synthesize coproporphyrin III from porphobilinogen (Heath & Hoare, 1959*a*, *b*). It was also shown that under certain conditions porphobilinogen was converted into uroporphyrin I and uroporphyrin III and that uroporphyrinogens I and III were decarboxylated to yield, ultimately, corpoporphyrins I and III respectively (Hoare & Heath, 1958).

The recent work of Bogorad (1958*a*, *b*, *c*) with enzyme preparations from spinach, wheat germ and *Chlorella*, and that of Granick & Mauzerall (1958) and Mauzerall & Granick (1958) with enzyme preparations from chick and rabbit reticulocytes, has shown that at least three enzymes are involved in the conversion of porphobilinogen into coproporphyrin III. Porphobilinogen deaminase purified from spinach extracts (Bogorad, 1958*a*) converts porphobilinogen, via unknown intermediates, into uroporphyrinogen I. In a preliminary report Lockwood & Rimington (1957) described the partial purification of an enzyme from chick erythrocytes which converted porphobilinogen into uroporphyrin III: the enzyme was called porphobilinogenase. When porphobilinogenase was preheated to 55° for 15 min. it catalysed the formation of uroporphyrin I from porphobilinogen. Similar effects of preheating had been observed earlier with crude preparations of *Chlorella* (Bogorad & Granick, 1953) and erythrocyte systems (Booij & Rimington, 1957). Similar investigations were reported in more detail by Granick & Mauzerall (1958), who purified a porphobilinogenase from chick erythrocytes and from rabbit reticulocytes. They established that uroporphyrinogen III was the reaction product in unheated preparations and uroporphyrinogen I was formed when preheated preparations were incubated with porphobilinogen. Uroporphyrinogen isomerase isolated from extracts of wheat germ (Bogorad, 1958*b*) catalysed the formation of uroporphyrinogen III from porphobilinogen in the presence of porphobilinogen deaminase; it had no action on uroporphyrinogen I or on porphobilinogen alone. Crude preparations of *Chlorella* were found to catalyse the formation of coproporphyrin from uroporphyrinogen (Bogorad, 1958*c*). Mauzerall & Granick (1958) partially purified a uroporphyrinogen decarboxylase from chick and rabbit

* Part 1: Heath & Hoare (1959*b*).

† Present address: A.R.C. Unit for Microbiology, University of Sheffield, Sheffield 10, Yorks.



Scheme 1

reticulocytes. The reactions involved in the biosynthesis of coproporphyrin III from porphobilinogen may be illustrated as in Scheme 1.

We have now found that the two enzymes porphobilinogen deaminase and uroporphyrinogen decarboxylase can be separated from one another in preparations derived from *R. spheroides*. Furthermore, an assay has been devised for uroporphyrinogen decarboxylase with porphobilinogen as substrate in the presence of an excess of porphobilinogen deaminase. Some properties of the two enzymes are described.

MATERIALS AND METHODS

Growth and harvest of organisms. Rhodopseudomonas spheroides was grown semi-anaerobically as previously described (Heath & Hoare, 1959*b*). The harvested cells were treated with cold acetone, as previously described, and the dried cells were stored at $-15°$.

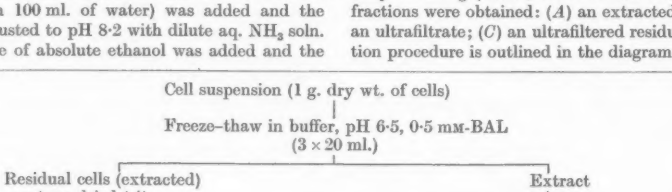
Substrates. Porphobilinogen (PBG) and uroporphyrins I and III were provided by Professor C. Rimington, F.R.S. Uroporphyrins II and IV were provided by Dr S. F. MacDonald.

Preparation of uroporphyrinogens. Uroporphyrins (500–1000 μg./ml. in 0·1 N-NaOH) were reduced with a large excess (2 g.) of freshly prepared 2·5% (w/w) sodium amalgam by a similar procedure to that used by Mauzerall & Granick (1958). The porphyrins were reduced in the dark in an atmosphere of N_2 and when completely colourless the mixture was filtered through three layers of Whatman no. 42 paper into a tube shielded from the light and immersed in a freezing mixture. The tube contained solid sodium dithionite and cysteine hydrochloride to make a final concentration of 1 mM each. The filtrate was adjusted to pH 6·8 with acetic acid or with phosphoric acid and was made up to 2 ml. In some cases, as specified in the results, sodium dithionite and cysteine hydrochloride were omitted. Under ideal conditions the uroporphyrinogen preparations were colourless and non-fluorescent, although oxidation occurred to a small degree in some cases during the pH adjustment.

Preparation of stimulating factor from liver. Fresh pig liver (135 g. wet wt.) was homogenized with 500 ml. of water in a Waring Blendor. The homogenate was boiled, and cooled and strained through muslin, producing a turbid yellow extract. The extract was boiled and filtered. Barium acetate (50 g. in 100 ml. of water) was added and the mixture was adjusted to pH 8·2 with dilute aq. NH_3 soln. An equal volume of absolute ethanol was added and the precipitate which formed was allowed to settle out overnight at $-15°$. The bulk of the supernatant was decanted off and the precipitate was collected by centrifuging, and was washed once with 50% (v/v) ethanol. The precipitate was suspended in water (about 10 ml.) and 3 ml. of 2 N-H_2SO_4 was added dropwise with continuous stirring. The suspension was centrifuged, producing a clear yellow supernatant. The residue was re-suspended in water and was re-extracted with 2 N-H_2SO_4 until the final extract (the fifth extraction) was almost colourless. The combined extracts were adjusted to pH 6·5 with 2 N-NaOH and were centrifuged to produce a bright-yellow solution. The solution was made up to 130 ml. (0·1 ml. = 100 mg. wet wt. of liver) and was stored at $-15°$. This is referred to as 'pig-liver extract' in all the experiments described.

Preparation and purification of enzymes. Acetone-dried cells (2 g.) were suspended in 50 ml. of ice-cold McIlvaine citrate–phosphate buffer, pH 6·5, diluted 1:10 with glass-distilled water containing 0·5 mM-dimercaptopropanol (BAL). This buffer was used throughout these investigations unless stated otherwise. The suspension was stirred in the cold for 3 hr. and was then frozen overnight. The frozen suspension was thawed out and centrifuged for 15 min. at 12 000*g*. The slightly opalescent red–brown supernatant was decanted off and the residual cells were resuspended in 10 ml. of buffer containing 0·5 mM-BAL. The suspension was centrifuged as before and the two supernatants were combined (total volume 35–45 ml.). The cell-free extract thus obtained was fractionated with solid $(NH_4)_2SO_4$, care being taken to keep the mixture cool (0–2°) by immersion in a freezing mixture. Ammonium sulphate was added in small quantities; the cell-free extract was stirred mechanically. Insoluble proteins were centrifuged for 15 min. at 12 000*g* and were dissolved or suspended in 10–20 ml. of buffer containing 0·5 mM-BAL. The different protein fractions were then ultrafiltered overnight, reducing the volume to less than 1 ml. The ultrafiltered fractions were made up to 2·5 ml. with buffer containing 0·5 mM-BAL and were centrifuged for 15 min. at 12 000*g* to remove small amounts of insoluble proteins. All enzyme fractions were stored at $-15°$.

Fractionation of whole acetone-dried cells for investigations on a stimulating factor for coproporphyrin formation. Acetone-dried cells were suspended in buffer, pH 6·5, containing 0·5 mM-BAL and were extracted by freezing and thawing three times successively with 20 ml. of buffer/g. dry wt. of cells. The residual cells were acetone-dried and the extract was concentrated by ultrafiltration through dialysis tubing (Grant, Rowe & Stanworth, 1958). Three fractions were obtained: (*A*) an extracted cell residue; (*B*) an ultrafiltrate; (*C*) an ultrafiltered residue. The fractionation procedure is outlined in the diagram (Scheme 2).


Cell suspension (1 g. dry wt. of cells)
Freeze–thaw in buffer, pH 6·5, 0·5 mM-BAL (3 × 20 ml.)
Residual cells (extracted) acetone-dried (A)
Extract
Ultrafiltration
Ultrafiltrate concentrated to 5 ml. (0·1 ml. = 20 mg. of cells) (B)
Ultrafiltered residue made to 5 ml. (0·1 ml. = 20 mg. of cells) (C)

Scheme 2

Incubation conditions. The standard conditions for most experiments included a reaction mixture of 0·5 ml. total volume, which was incubated for 2 hr. in an atmosphere of N_2. Anaerobic conditions were maintained by carrying out the reactions in small centrifuge tubes, which were inserted into Thunberg tubes containing alkaline pyrogallol. The Thunberg tubes were then alternately evacuated and flushed with O_2-free N_2 (three times). Incubations were carried out at 37° in the dark by covering the incubation bath with a black cloth.

Estimation of porphyrins. The procedure previously used (Heath & Hoare, 1959*b*) was slightly modified. One drop of iodine (1%, w/v, in absolute ethanol) was added to each reaction mixture at the end of the incubation period, and this was followed within 10–15 sec. by one drop of 1% (w/v) sodium thiosulphate. Ethyl acetate–acetic acid mixture (3:1, v/v; 5 ml.) was then added and the mixture was centrifuged; the residue was re-extracted with 5 ml. of ethyl acetate–acetic acid mixture and the combined extracts were transferred to separating funnels. The uroporphyrin was extracted with saturated sodium acetate followed by 3% (w/v) sodium acetate as previously described. The combined sodium acetate extracts were acidified to pH < 1·0 with conc. HCl and were made up to a given volume (generally 75 ml.) with 5% (w/v) HCl and the extinctions at 380, 406 and 430 mμ were measured in a Unicam SP. 500 spectrophotometer. Coproporphyrin was then extracted directly from the ethyl acetate phase with 10% (w/v) HCl; the extinction of the combined acid extracts, made up to 25 ml. or 75 ml. with 10% (w/v) HCl, was read at 380, 402 and 430 mμ. The correction factors of Rimington & Sveinsson (1950) were used to calculate the porphyrin concentrations.

Direct assay of porphobilinogen deaminase. Preparations of porphobilinogen deaminase which had been found to be free from uroporphyrinogen decarboxylase were assayed directly by the following procedure. Reaction mixtures of 0·5 ml. total volume contained PBG (100 μg.) in McIlvaine's citrate–phosphate buffer at pH 7·6. Incubations were carried out anaerobically for 1 hr. at 37°, after which the reaction mixture was treated with iodine and thiosulphate as before, diluted to 25 ml. (or 75 ml.) with 5% (w/v) HCl and examined directly in a spectrophotometer. The small amount of protein present did not interfere.

Enzyme activities were expressed as μg. of porphyrin formed/mg. of protein/hr. Protein estimations were carried out by the method of Lowry, Rosebrough, Farr & Randall (1951), bovine-serum albumin being used as standard.

Paper chromatography of free porphyrins and of porphyrin esters. Porphyrin esters, prepared by esterification with 5% (v/v) H_2SO_4 in methanol, were chromatographed by the method of Falk & Benson (1953). Free porphyrins were chromatographed by the method of Falk, Dresel, Benson & Knight (1956) and by the modified procedure of Eriksen (1958).

Column chromatography of porphyrin esters. Chromatography on magnesium oxide grade III and on alumina grade IV was carried out according to the procedure of Nicholas (1951). Small columns of 1 cm. diam. and 12 cm. length were used.

Paper electrophoresis of porphyrins. Free porphyrins were applied to the cathode end of a strip of Whatman no. 3 paper (4 cm. width, 60 cm. length) equilibrated with 1% (w/v) anhydrous Na_2CO_3 and 0·1 mM-disodium ethylenediaminetetra-acetate. A current of 3 mA, 240 v was applied for 2–3 hr.; the migration of porphyrins could be observed visually. Uroporphyrin and coproporphyrin were run simultaneously as markers.

RESULTS

Ammonium sulphate fractionation of cell-free extracts

Cell-free extracts were prepared from acetone-dried cells as described in the Materials and Methods section. As has already been shown (Heath & Hoare, 1959*b*), the relative amounts of uroporphyrin and coproporphyrin formed by acetone-dried cells and by cell-free extracts depend upon the protein concentration and the time of incubation. It is thus difficult to assess the degree of extraction of the respective porphyrin-forming enzymes. In a typical experiment 2 g. of acetone-dried cells after extraction yielded 40 ml. of cell-free extract containing a total of 320 mg. of protein, representing 16% of the starting material. The enzyme activities are shown in Table 1.

The above-mentioned cell-free extract was fractionated with $(NH_4)_2SO_4$ and, after overnight ultrafiltration, the protein content of each fraction was determined before and after removing insoluble matter by centrifuging. Each fraction was then assayed anaerobically under the standard conditions (2 hr. at 37°). The distribution of protein

Table 1. *Extraction of protein and porphyrin-forming enzyme activity from acetone-dried cells of* Rhodopseudomonas spheroides

Acetone-dried cells, or cell-free extract, were incubated anaerobically in a total volume of 0·5 ml. with PBG (100 μg.) for 2 hr. at 37° in citrate–phosphate buffer, pH 6·5.

	Dry wt. per assay (mg.)	Porphyrin formed (μg./2 hr.)		Enzyme activity (μg. of porphyrin/mg./hr.)		
		Uro-	Copro-	Uro-	Copro-	Total
Acetone-dried cells	2	8	2	2·0	0·5	2·5
	5	19	8	1·9	0·8	2·7
	10	28	20	1·4	1·0	2·4
Cell-free extract	1·6	40	9	12·5	2·8	15·3

Table 2. *Distribution of protein and porphyrin-forming enzyme activity in ammonium sulphate fractions from cell-free extracts of* Rhodopseudomonas spheroides

Cell-free extract (40 ml., 300 mg. of protein) was fractionated with ammonium sulphate; each fraction was ultrafiltered overnight as described in the text and assayed for porphyrin-forming enzyme activity.

$(NH_4)_2SO_4$ fraction (%, w/v)	Total protein (mg.)	Soluble protein (mg.)	Protein per assay (mg.)	Porphyrin formed (μg./2 hr.)		PBG deaminase activity* (μg. of uro-/mg./hr.)
				Uro-	Copro-	
0–15	80	—		Discarded		—
15–30	100	85	0·5	44	2	44
30–35	40	30	0·8	27	2	17
35–40	20	17	0·4	1	2	1
40–45	17	17	0·3	0	0·5	Nil

* Assayed at pH 6·5, which is not the optimum pH.

Table 3. *Ammonium sulphate subfractionation of porphobilinogen deaminase*

Starting material (34 ml.) contained 442 mg. of protein of enzyme activity 17 μg. of uro-/mg. of protein/hr., total enzyme units 7600. Solid ammonium sulphate was added to the percentage (w/v) shown below; each precipitate was dissolved in buffer, pH 6·5, containing 0·5 mM-BAL, and was assayed for porphyrin-forming enzyme activity. Enzyme unit: mg. of protein catalysing the formation of 1 μg. of uroporphyrin/hr. under the assay conditions.

$(NH_4)_2SO_4$ fraction (%, w/v)	Total protein (mg.)	Protein per assay (mg.)	Enzyme activity (μg. of uro-/mg./hr.)	Enzyme units
0–15	105	0·5	2	210
15–22	119	0·6	8·5	1012
22–30	166	0·4	36·2	6009
30–36	38	0·7	17·2	654

Table 4. *Indirect assay of uroporphyrinogen decarboxylase in ammonium sulphate fractions from* Rhodopseudomonas spheroides

Test fraction was incubated anaerobically in a total volume of 0·5 ml. with an excess of PBG deaminase (0·6 mg. of protein), pig-liver extract (0·1 ml.) and PBG (100 μg.) for 2 hr. at 37° in citrate–phosphate buffer, pH 6·5.

$(NH_4)_2SO_4$ fraction (%, w/v)	Protein per assay (mg.)	Copro-porphyrin formed (μg./2 hr.)	Decarboxylase activity (μg. of copro-/mg./hr.)
35–40	0·4	6·4	8
40–45	0·33	5·1	7·7

and enzyme activity in the different fractions is shown in Table 2. The fraction precipitating between 0 and 15 % (w/v) of $(NH_4)_2SO_4$ was grey–green and redissolved only incompletely in buffer solution; in every case when tested it was devoid of enzyme activity. Porphobilinogen deaminase activity was found in the protein fractions precipitating between 15 and 35 % (w/v) of $(NH_4)_2SO_4$. Protein fractions precipitating between 45 and 55 % of $(NH_4)_2SO_4$ were pink and a determination of the absorption spectrum at pH 6·5 showed sharp peaks at 415, 520 and 548 mμ, corresponding to a reduced cytochrome *c* (cf. Vernon & Kamen, 1954). Fractions containing PBG deaminase activity from several fractionation procedures applied to different batches of acetone-dried cells were combined and subfractionated with $(NH_4)_2SO_4$. Such a subfractionation is illustrated in Table 3. Nearly 80 % of the PBG deaminase activity precipitated between 15 and 30 % (w/v) of $(NH_4)_2SO_4$. It was completely free from decarboxylating enzymes forming coproporphyrin.

Indirect assay of uroporphyrinogen decarboxylase. Protein fractions precipitated by $(NH_4)_2SO_4$ concentrations greater than 35 %, and which formed very little coproporphyrin from PBG, were tested for uroporphyrinogen decarboxylase by an indirect method with PBG as substrate. Use was made of the PBG deaminase preparations from earlier $(NH_4)_2SO_4$ fractions (15–35 %), particularly after a second subfractionation, as just described, to free them from coproporphyrin-forming enzymes. On incubating test fractions with PBG, together with an excess of PBG deaminase, the formation of coproporphyrin indicated the presence of uroporphyrinogen decarboxylase. Protein fractions precipitated by $(NH_4)_2SO_4$ concentrations above 35 % (w/v) (as shown in Table 2) were tested in this way (Table 4).

Protein fractions containing uroporphyrinogen decarboxylase free from PBG deaminase were stable on storage at −15° for 2–3 weeks in the presence of 0·5 mM-BAL (Table 5). When a number

Table 5. *Stability tests on uroporphyrinogen decarboxylase preparations*

Uroporphyrinogen decarboxylase was assayed by the indirect method, in two preparations after storage at −15°.

Enzyme preparation	Age of preparation (days)	Protein per assay (mg.)	Decarboxylase activity (μg. of copro-/mg./hr.)
D4 [35–40% (w/v) $(NH_4)_2SO_4$]	Fresh	0·34	10
	7	—	17
	20	—	12
C5 [36–40% (w/v) $(NH_4)_2SO_4$]	Fresh	0·44	9
	13	—	11
	26	—	12

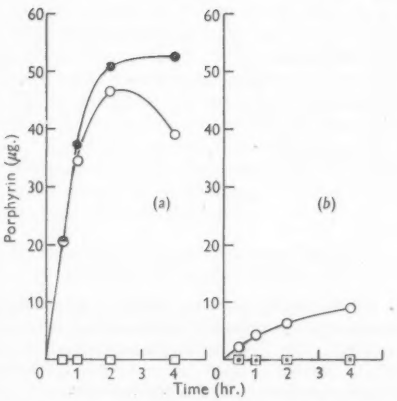


Fig. 1. Effect of time of incubation on the formation of uroporphyrin (*a*) and coproporphyrin (*b*) in the indirect assay of uroporphyrinogen decarboxylase. PBG (100 μg.) was incubated anaerobically in citrate–phosphate buffer, pH 6·5, with an excess of PBG deaminase (1600 μg. of protein) (●), or uroporphyrinogen decarboxylase (400 μg. of protein) (□), or uroporphyrinogen decarboxylase together with an excess of PBG deaminase (○), in a final volume of 0·5 ml. at 37° for the times indicated.

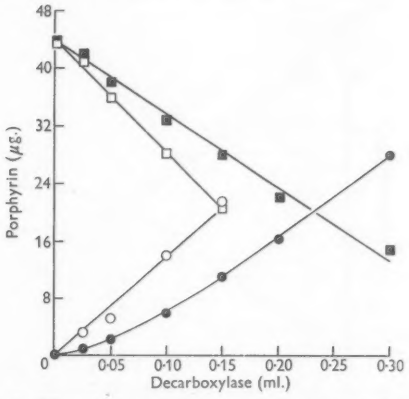


Fig. 2. Effect of enzyme concentration on porphyrin formation in the indirect assay of uroporphyrinogen decarboxylase, and the effect of adding pig-liver extracts to the system. PBG (100 μg.) was incubated for 2 hr. at 37° anaerobically at pH 6·5, with an excess of PBG deaminase (570 μg. of protein) and different amounts of uroporphyrinogen decarboxylase (7400 μg. of protein/ml.) in a final volume of 0·5 ml. with and without 0·1 ml. of pig-liver extract (prepared as described in the Materials and Methods section). ●, Coproporphyrin formation in the absence of pig-liver extract; ○, coproporphyrin formation in the presence of pig-liver extract; ■, uroporphyrin formation in the absence of pig-liver extract; □, uroporphyrin formation in the presence of pig-liver extract.

of stored decarboxylase preparations were combined and subfractionated with $(NH_4)_2SO_4$, no purification was achieved and the treated preparations lost activity after storage for 2–3 days at −15°. Instability of the decarboxylase was also shown in the early stages of enzyme extraction. When attempts were made to improve the degree of extraction of acetone-dried cells by homogenizing in buffer in a Potter–Elvehjem glass homogenizer, the resulting extracts formed only uroporphyrin from PBG. Inactive preparations could not be reactivated by the addition of BAL.

A progress curve for coproporphyrin formation and uroporphyrin formation in the indirect assay is illustrated in Fig. 1. Control curves show that no coproporphyrin is formed from PBG by the decarboxylase or deaminase preparations alone.

Fig. 2 illustrates the increase in coproporphyrin production with increasing concentration of uroporphyrinogen decarboxylase in the indirect-assay procedure; the uroporphyrin concentration correspondingly decreases.

Tests with uroporphyrinogens as substrates. Uroporphyrinogens were prepared as described in the Materials and Methods section. When tested as substrates with a partially purified decarboxylase preparation which had no effect on PBG, coproporphyrin was formed. The time course for the reaction, with uroporphyrinogen III as substrate, over a 4 hr. incubation period is shown in Fig. 3.

With a fixed concentration of uroporphyrinogen (prepared in the presence of cysteine and dithionite) and different concentrations of decarboxylase, the amount of coproporphyrin formed per hour was proportional to the enzyme concentration (Fig. 4).

Uroporphyrinogens I, II and IV were also decarboxylated to yield coproporphyrins. A direct comparison of the rates of reaction with the four uroporphyrinogens was not made. In the absence of sodium dithionite, the rate of coproporphyrin formation was independent of uroporphyrinogen III concentration over the range 8 μM–0·16 mM.

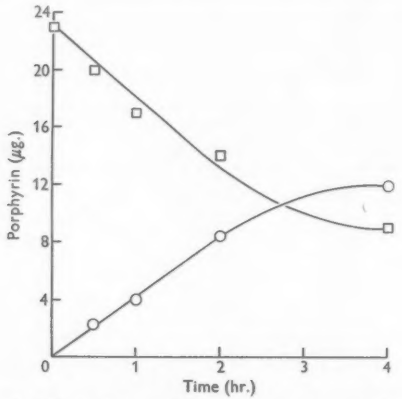


Fig. 3. Effect of time of incubation on coproporphyrin formation from uroporphyrinogen III. Coproporphyrin formation (○) and uroporphyrin recovered (□) when uroporphyrinogen decarboxylase (430 μg. of protein) was incubated anaerobically with uroporphyrinogen III (23 μg.) and pig-liver extract (0·1 ml.) at pH 6·5 in a final volume of 0·5 ml. at 37° for the times indicated.

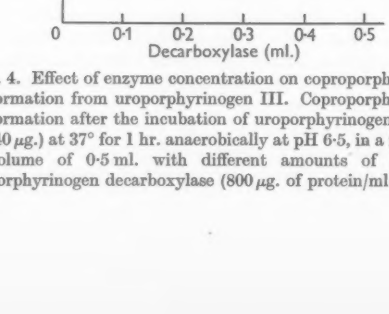


Fig. 4. Effect of enzyme concentration on coproporphyrin formation from uroporphyrinogen III. Coproporphyrin formation after the incubation of uroporphyrinogen III (40 μg.) at 37° for 1 hr. anaerobically at pH 6·5, in a final volume of 0·5 ml. with different amounts of uroporphyrinogen decarboxylase (800 μg. of protein/ml.).

Properties of partially purified porphobilinogen deaminase

pH optimum was determined in McIlvaine's citrate–phosphate buffer over the range pH 6·4–8·0; borate buffer was used up to pH 8·8. For 1 hr.

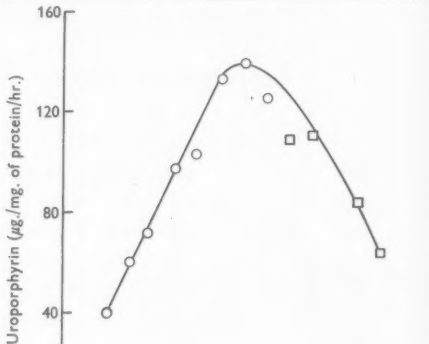


Fig. 5. pH–activity curve for PBG deaminase. PBG deaminase (160 μg. of protein) was incubated anaerobically with PBG (100 μg.) in a final volume of 0·5 ml. for 1 hr. at 37° in different buffers (pH 6·4–9·0). ○, McIlvaine citrate–phosphate buffer; □, borate buffer.

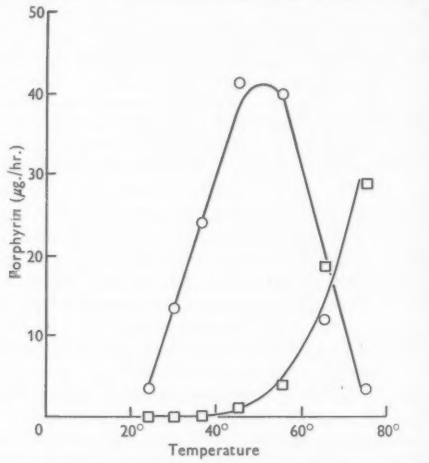


Fig. 6. Optimum temperature for PBG deaminase. PBG (100 μg.) was incubated anaerobically for 1 hr. at pH 7·6 in a final volume of 0·5 ml. at the temperatures indicated: ○, with PBG deaminase (190 μg. of protein); □, without PBG deaminase. Figures with PBG deaminase are corrected for uroporphyrin formed non-enzymically.

incubations anaerobically at 37° the optimum pH was 7·6 (Fig. 5).

At pH 7·6 the rate of uroporphyrin formation over a 1 hr. incubation period was measured at different temperatures. Both the enzymic and non-enzymic reactions were studied. An optimum temperature between 45 and 55° was observed under these conditions (Fig. 6). A re-determination of enzyme activity at four different pH values at 55° showed an optimum pH at 7·0. The following activities (μg. of uroporphyrin formed/mg. of protein/hr.) were found: 248 at pH 6·5, 279 at pH 7·0, 216 at pH 7·6, 205 at pH 8·0.

In the determination of the Michaelis constant, the PBG concentration was varied over the range 5 μM–0·1 mM. The volume of the reaction mixture was increased to 20 ml. to ensure that a measurable quantity of porphyrin was produced, leaving sufficient PBG unreacted at the end of the incubation period. K_m 40 μM was calculated from a plot of S/V against S (Fig. 7). This value was unchanged by the presence of porphobilinogen α-carboxylic acid (50 μM) in the reaction mixture.

Investigations on a stimulating factor for formation of coproporphyrin

We have previously reported evidence for the presence of a heat-stable factor which stimulates coproporphyrin formation from PBG (Hoare & Heath, 1958; Heath & Hoare, 1959*a*). When extracts of acetone-dried cells were ultrafiltered, formation of coproporphyrin from PBG was diminished but was partially restored by adding the ultrafiltrate. The ultrafiltrate was heat-stable (10 min. at 100°, pH 6·5). Similar stimulating activity was found in ultrafiltrates of chick blood and in extracts of pig liver and of baker's yeast. Since liver was found to be a convenient source of stimulating activity, preparations were made from this according to the procedure given in the Materials and Methods section. Preparations were stored at −15° and a single preparation stored over several months was used in all experiments.

Stimulating effects with crude enzyme preparations. Acetone-dried cells were extracted and resolved into three fractions as described in the Materials and Methods section. When assayed anaerobically the extracted cell residue (*A*) had very low activity, as also had the ultrafiltrate (*B*). The ultrafiltered

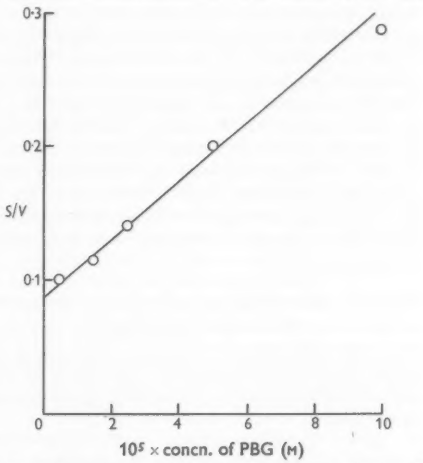


Fig. 7. Lineweaver–Burk plots. Reaction mixtures, of 20 ml. final volume, containing PBG deaminase (780 μg. of protein) were incubated at 37°, pH 7·6, for 30 min. in open tubes with the concentrations of PBG indicated. *V*, Uroporphyrin formed (μg./tube in 30 min. at 37°, pH 7·6). *S*, 10^5 × molar concn. of PBG.

Table 6. *Stimulation of coproporphyrin formation by ultrafiltrates from cell-free extracts of* Rhodopseudomonas spheroides

Cell fractions (equivalent to 20 mg. of whole cells), prepared as described in the text, were incubated in a total volume of 0·5 ml. with PBG (100 μg.) at 37° in citrate–phosphate buffer, pH 6·5. Heated preparations were kept at 100°, pH 6·5, for 10 min. Expt. I: anaerobic incubation for 2 hr. Expt. II: aerobic incubation (in tubes open to the air, without shaking) for 4 hr. Expts. I and II were carried out with different batches of cells.

	Porphyrin formed			
	Expt. I (anaerobic)		Expt. II (aerobic)	
Test system	Uro- (μg.)	Copro- (μg.)	Uro- (μg.)	Copro- (μg.)
Extracted cell residue (*A*)	3	—	10	—
Ultrafiltrate (*B*)	—	—	—	—
Ultrafiltered residue (*C*)	28	6	36	7
(*C*) + (*A*)	15	16	24	11
(*C*) + (*B*)	17	22	20	18
(*A*) + (*B*) + (*C*)	10	18	15	24
(*A*) heated + (*B*) + (*C*)	10	17	20	16
(*A*) + (*B*) heated + (*C*)	12	18	15	24

extract (*C*) formed mainly uroporphyrin with some coproporphyrin, and on addition of the ultrafiltrate there was a stimulation of coproporphyrin formation and a decrease in uroporphyrin (Table 6). The stimulating effect of the ultrafiltrate was unchanged after heating for 10 min. at 100°, pH 6·5. The extracted cell residue (*A*), although forming very small amounts of uroporphyrin from PBG, also stimulated coproporphyrin production when added to the ultrafiltered extract (*C*). Again, the stimulating activity of fraction (*A*) was heat-stable, and since no further stimulation was obtained by the combined actions of fraction (*A*) and the ultrafiltrate (*B*), it was concluded that a single stimulating factor was present in both fractions. When uroporphyrinogen was tested as substrate in the ultrafiltered system a similar stimulation of coproporphyrin formation was observed (Table 7). Under aerobic conditions, however, all three fractions (*A*, *B* and *C*) were necessary for maximum coproporphyrin formation from PBG (Table 6). The additional stimulating effect of the cell residue (*A*) under these aerobic conditions was destroyed by heating to 100° for 10 min. at pH 6·5. The stimulating effect of the ultrafiltrate is not due to any protecting effect on uroporphyrinogen since, after anaerobic incubation in the dark at 37° in the absence of enzymes, the same amount of uroporphyrin was recovered in the presence as in the absence of the ultrafiltrate; in both cases non-fluorescent pink pigments were formed in addition to uroporphyrin. The stimulating effect of the ultrafiltrate does not appear to be due to sulphydryl compounds since its activity was stable towards iodine oxidation.

Stimulating activity in cell ultrafiltrates was strongly adsorbed on charcoal and on alumina. It was precipitated, or adsorbed, with insoluble barium salts at pH 8·0. Thiamine pyrophosphate (0·1 mM–10 μM) with or without magnesium or manganous chloride (0·1 mM–10 μM) failed to stimulate coproporphyrin formation by the ultrafiltered enzyme preparations. Ultrafiltrates were completely inactivated by ashing; they were stable to weak acid or alkali at room temperature, but were inactivated by hot concentrated HCl and aq. NH_3 soln. (sp.gr. 0·880). All attempts to adsorb and elute activity from ion-exchange resins were unsuccessful.

Stimulating effects with partially purified enzyme fractions. When PBG deaminase and uroporphyrinogen decarboxylase were separated by fractionation with $(NH_4)_2SO_4$, the stimulating effect of the pig-liver extract was re-examined. In the 'indirect assay', with PBG as substrate, a definite stimulation of coproporphyrin formation, with a corresponding decrease in uroporphyrin, was observed (see Fig. 2).

Attempts to demonstrate a stimulation of coproporphyrin formation by the pig-liver extract, with uroporphyrinogen as substrate, with the partially purified decarboxylase preparation have not yielded very conclusive results. A single batch of acetone-dried cells was used to provide a cell-free extract

Table 7. *Stimulation of coproporphyrin formation from uroporphyrinogen*

Ultrafiltered extract (equivalent to 20 mg. of whole cells) was incubated anaerobically in a total volume of 0·5 ml. with uroporphyrinogen for 2 hr. at 37° in citrate–phosphate buffer, pH 6·5.

Test system	Porphyrin formed (μg.) Uro-	Copro-
Uroporphyrinogen + ultrafiltered extract	42	20
Uroporphyrinogen + ultrafiltered extract + ultrafiltrate	10	52
Uroporphyrinogen control (no enzyme)	74	—

Table 8. *Stimulation of coproporphyrin production from uroporphyrinogen III or from porphobilinogen by the addition of a pig-liver extract to crude and partially purified preparations from* Rhodopseudomonas spheroides

Enzyme preparations were made as described in the text: 'crude enzyme' (540 μg. of protein), 'purified enzyme 1' (530 μg. of protein) or 'purified enzyme 2' (1100 μg. of protein). These were incubated anaerobically in a total volume of 0·5 ml. with uroporphyrinogen III (100 μg.) or with PBG (100 μg.) in the presence of an excess of PBG deaminase (1300 μg. of protein), with or without a pig-liver extract (0·1 ml.), for 18 hr. at 37° in citrate–phosphate buffer, pH 6·5.

Test system	Substrate	Porphyrin formed (μg.) Uro-	Copro-
Crude enzyme	Uroporphyrinogen III	77	6
Crude enzyme + pig-liver extract	Uroporphyrinogen III	69	11
Purified enzyme 1	Uroporphyrinogen III	58	24
Purified enzyme 1 + pig-liver extract	Uroporphyrinogen III	47	27
Purified enzyme 2	Uroporphyrinogen III	68	8
Purified enzyme 2 + pig-liver extract	Uroporphyrinogen III	68	11
Purified enzyme 1 + PBG deaminase	PBG	18	34
Purified enzyme 1 + PBG deaminase + pig-liver extract	PBG	3	49

('crude enzyme') part of which was retained and ultrafiltered directly; the remainder was fractionated with $(NH_4)_2SO_4$. A fraction ('purified enzyme 1') precipitating between 40 and 45% (w/v) of $(NH_4)_2SO_4$ was found to contain uroporphyrinogen decarboxylase free from PBG deaminase. A fraction ('purified enzyme 2'), precipitating between 45 and 55% (w/v) of $(NH_4)_2SO_4$, was also found to have a very weak uroporphyrinogen decarboxylase activity and was free from PBG deaminase. Both these fractions and the original cell-free extract were ultrafiltered twice, from 20 ml. of solution in buffer, pH 6·5, containing 0·5 mM-BAL, to a volume less than 1 ml. The ultrafiltered preparations were then tested for coproporphyrin formation from uroporphyrinogen both in the presence and in the absence of pig-liver extract. The results are shown in Table 8.

Examination of the porphyrin reaction products

The porphyrins formed under different incubation conditions were examined by chromatographic techniques. It has been noted before that whole fresh acetone-dried cells form largely the III series isomers of uroporphyrin and coproporphyrin from PBG. There was no evidence for the formation of porphyrins with less than four carboxyl groups. Cell-free extracts formed both the I and III series isomers, the I series isomer predominating in most cases. With both the whole acetone-dried cells and the cell-free extracts there was evidence for the formation of intermediate penta-, hexa- or hepta-carboxylic porphyrins. After fractionation with $(NH_4)_2SO_4$ the fractions containing PBG deaminase formed uroporphyrin I and no trace of any other porphyrin. In the presence of the partially purified decarboxylase fraction, coproporphyrin I and intermediate porphyrins were formed in addition.

Porphyrin fractions estimated as 'coproporphyrin' according to the procedure given in the Materials and Methods section, on chromatography by the Eriksen method (1958), showed coproporphyrin I together with traces of coproporphyrin III in some cases; no other porphyrins were present. Acid extracts containing coproporphyrin, from a large number of incubations, were esterified with methanol–H_2SO_4 (95:5, v/v). Coproporphyrin ester was extracted into $CHCl_3$ and was washed successively with water, aq. 2N-NH_3 soln. and water. Typical crystals of coproporphyrin I tetramethyl ester were formed on concentration of the washed $CHCl_3$ extract. Insufficient material was available for recrystallization and determination of m.p. The combined coproporphyrin methyl esters were eluted as a single sharp band from a column of alumina grade IV by benzene–$CHCl_3$ (1:1, v/v).

Extracts containing uroporphyrin were concentrated to dryness, were similarly esterified and the methyl esters were chromatographed on paper (Falk & Benson, 1953). One or two spots with R_F greater than that for uroporphyrin III octamethyl ester were invariably found in addition to a strong spot corresponding to uroporphyrin I.

When the esters were hydrolysed and the free porphyrins were chromatographed by the method of Falk *et al.* (1956), in addition to uroporphyrin there were two well-defined fluorescent spots intermediate between uroporphyrin and coproporphyrin. The faster R_F component was present in much higher concentrations. In a typical chromatogram in which uroporphyrin, coproporphyrin and deuteroporphyrin were run simultaneously as markers, the following R_F values were found: deuteroporphyrin, 0·83; coproporphyrin III, 0·51; major unknown, 0·29; minor unknown, 0·10; uroporphyrin I, 0·012. The uroporphyrin always appeared as a double spot, the faster R_F component being yellow. This

Table 9. *Chromatographic separation of porphyrin reaction products*

Combined 'uroporphyrin fractions' from a number of reaction mixtures were esterified and the methyl esters were chromatographed on a column of magnesia grade III; eluates were concentrated to dryness and were hydrolysed in 6N-HCl; the free porphyrins were chromatographed by the method of Falk, Dresel, Benson & Knight (1956).

Magnesia chromatogram		Paper chromatogram
Band	Eluting solvent	R_F values
(1) Sharp and strong	$CHCl_3$–0·5% methanol	0·05 (Uroporphyrin) 0·08 [Pseudo-uroporphyrin (yellow)] 0·17 (Trace of unknown intermediate)
(2) Diffuse band	$CHCl_3$–0·5% methanol	0·17 (Minor intermediate) 0·30 (Major intermediate)
(3) Distinct band	$CHCl_3$–1% methanol	0·33 (Major intermediate)
(4) No distinct band	$CHCl_3$–5% methanol	0·33 (Traces only)
(5) No distinct band	$CHCl_3$–50% methanol	0·50 (Traces of coproporphyrin) 0·033 (Uroporphyrin I marker) 0·47 (Coproporphyrin I marker)

may correspond to the 'pseudo-uroporphyrin' reported by Falk *et al.* (1956).

Similar intermediate porphyrins were observed with the partially purified decarboxylase and uroporphyrinogen as substrate. Controls with uroporphyrinogen alone showed only uroporphyrin on chromatography although other non-fluorescent pink pigments were present: these may be related to the pigments formed from PBG under certain conditions (Brockman & Gray, 1953).

Paper electrophoresis of intermediate porphyrins. 'Uroporphyrin fractions' were resolved into five components travelling as follows: uroporphyrin together with pseudo-uroporphyrin, 8–10 cm.; minor component, 5 cm.; major component, 2–3 cm.; very weak spot on origin (which may be due to traces of coproporphyrin), 0·5 cm.

Attempts to isolate intermediate porphyrins by column chromatography on magnesia. The 'uroporphyrin fractions' from a number of reaction mixtures were accumulated and esterified. The methyl esters were then chromatographed on a column of magnesium oxide grade III, mixtures of $CHCl_3$–methanol being used as the eluting solvent. The eluates were concentrated to dryness and were hydrolysed with 6N-HCl for 48 hr. at room temperature. The free porphyrins were then run on paper chromatograms in lutidine–water (Falk *et al.* 1956), with uroporphyrin I and coproporphyrin I as markers. The results are summarized in Table 9. No further characterization was possible owing to the small quantities of material available.

DISCUSSION

Mauzerall & Granick (1958) and Granick & Mauzerall (1958) in their studies on porphyrin biosynthesis by chick and rabbit reticulocytes were able to separate PBG deaminase from uroporphyrinogen decarboxylase by zone electrophoresis on starch plates at pH 7·65. These two enzymes have now been separated from one another in cell-free extracts of *R. spheroides* by fractional precipitation with ammonium sulphate. We have also applied a similar fractionation procedure to the strain of *R. capsulatus* found by Cooper (1956) to produce a coproporphyrin precursor under certain cultural conditions; this also effected a separation of PBG deaminase from uroporphyrinogen decarboxylase.

The development of an indirect assay for uroporphyrinogen decarboxylase with PBG as substrate in the presence of an excess of PBG deaminase greatly facilitated the following of activity of different preparations of uroporphyrinogen decarboxylase. The indirect assay was found to be reproducible and in this respect was far more satisfactory than the direct assay with uroporphyrinogens as substrates. Indeed assays of a single-enzyme preparation by both the direct and indirect methods did not agree (see, for example, Table 8). Activities in the direct assays with uroporphyrinogens were invariably lower. This may be attributed to the presence of inhibitors in the uroporphyrinogen preparations. It is unlikely to be due to traces of mercury from the sodium-amalgam reduction since the reduction was carried out under alkaline conditions. Lower activities were found independently of the presence or absence of cysteine or of dithionite with the uroporphyrinogen preparation. It was in many cases difficult to prevent absolutely any re-oxidation of the uroporphyrinogen preparations. It is quite likely that products of partial re-oxidation of uroporphyrinogens inhibit the decarboxylase. Non-fluorescent pink pigments invariably formed after re-oxidation of uroporphyrinogens and these unidentified products may also inhibit uroporphyrinogen decarboxylase.

Although a large number of enzyme fractionations were carried out on different batches of acetone-dried cells, the final partially purified enzyme preparations had very similar activities. Uroporphyrinogen decarboxylase activities ranged from 8 to 11 μg. of coproporphyrin formed/mg. of protein/hr. It is difficult to compare the activity of our preparations with those of Mauzerall & Granick (1958), who used 0·5 ml. of enzyme preparation containing about 0·1 mg. of nitrogen/ml., and found 2 μm-moles of coproporphyrin formed/hr. (These values are derived from the most active system shown in Fig. 9 of Mauzerall & Granick, 1958.) Assuming that their preparation contained 0·312 mg. of protein (equivalent to 0·05 mg. of nitrogen/0·5 ml. of enzyme) its activity would be 4·2 μg. of coproporphyrin formed/mg./hr. Our preparations of uroporphyrinogen decarboxylase were stable to storage at $-15°$ only in the presence of sulphydryl compounds such as BAL. This suggests that it is a sulphydryl enzyme. This is confirmed by preliminary experiments in which the enzyme was found to be inhibited by heavy metals (mercury and copper). The loss of decarboxylase activity when acetone-dried cells were homogenized in a Potter–Elvehjem glass homogenizer may be due to oxidation of essential sulphydryl groups. The few investigations on the decarboxylase from *R. spheroides* show that it is very similar in properties to the enzyme isolated from chick and rabbit reticulocytes by Mauzerall & Granick (1958). This extends to the observation that all four position-isomers of uroporphyrinogen are decarboxylated and also that the decarboxylation proceeds via the formation of intermediate hepta-, hexa- and penta-carboxylic porphyrins which have been detected chromatographically.

Table 10. *Comparison of porphobilinogen deaminase activities from different sources*

	Enzyme source	Activity as expressed by authors	Activity (μg. of uro-/mg. of protein/hr.)
Lockwood & Rimington (1957)	Chick blood	μg. of uro-/ml./hr. 133 μg. of uro-/9 mg. of protein/hr.	14·8
Granick & Mauzerall (1958)	Chick blood	μmole of PBG/ml. of enzyme/hr. 0·3 μmole of PBG/2·1 mg. of protein/hr.	29·8
Granick & Mauzerall (1958)	Rabbit reticulocytes	0·3 μmole of PBG/0·35 mg. of protein/hr.	178
Bogorad (1958*a*)	Spinach	1300 units/mg. of protein (1 unit: enzyme required to catalyse the consumption of 1 μm-mole of PBG/hr.)	270
Present paper	*R. spheroides*	μg. of uro-/mg. of protein/hr.	200*

* At pH 7·6, 45°.

The fact that intermediate porphyrins were invariably found in small amounts as products of the action of uroporphyrinogen decarboxylase and that these were extracted into the 'uroporphyrin fractions' raises the question of the validity of the figures quoted for uroporphyrin formation. It has been assumed that the contribution of intermediate porphyrins to the observed absorption could be neglected. However, it should be borne in mind that the uroporphyrin measured does include variable small quantities of other porphyrins. Cell-free extracts of *R. spheroides* are a very convenient source of PBG deaminase with a high specific activity. After only two fractionations with ammonium sulphate, preparations were obtained with an activity of the order of 200 μg. of uroporphyrin formed/mg. of protein/hr. This compares favourably with enzyme preparations from other sources (Table 10).

PGB deaminase from *R. spheroides* is similar in properties to the enzyme isolated from blood and from spinach. It is comparatively thermostable with a temperature optimum around 45–55° under the assay conditions employed in our experiments. Rather surprisingly there was no increase in the amount of PBG deaminase extracted from acetone-dried cells when the extraction was carried out at 50°.

Although opsopyrrole carboxylic acid has been shown to be a competitive inhibitor of PBG deaminase (Bogorad, 1957; Carpenter & Scott, 1959), porphobilinogen α-carboxylic acid had no affinity for the enzyme. This suggests that the enzyme-substrate linkage involves a free (unsubstituted) α-position on the pyrrole nucleus. The Michaelis constant of 40 μM found for the PBG deaminase from *R. spheroides* is very close to that reported for the spinach enzyme (Bogorad, 1958*b*).

The nature of the stimulating factor in pig-liver extracts is still obscure. Significant stimulations of coproporphyrin formation from PBG were consistently obtained both with crude cell-free extracts after ultrafiltration and with the partially purified preparation. However, when uroporphyrinogens were used as substrates, coproporphyrin formation was stimulated by the addition of pig-liver extract to crude cell-free extracts but not with the purified decarboxylase. The failure to get a significant stimulation of coproporphyrin formation from uroporphyrinogens with the partially purified decarboxylase may be due to the presence of inhibitors in the uroporphyrinogen preparations. In this case it must be assumed that the purified enzyme is more sensitive to inhibitors than is the crude enzyme in cell-free extracts. It has already been mentioned that uroporphyrinogen decarboxylase activities determined with uroporphyrinogens as substrates are lower than those found in the indirect assay with PBG and a common factor may be involved in these two effects.

SUMMARY

1. Porphobilinogen deaminase and uroporphyrinogen decarboxylase have been separated from each other in cell-free extracts of *Rhodopseudomonas spheroides* by fractionation with ammonium sulphate.

2. A reproducible 'indirect' assay for uroporphyrinogen decarboxylase has been devised with porphobilinogen as substrate with an excess of porphobilinogen deaminase.

3. The Michaelis constant, temperature optimum and pH optimum of the partially purified porphobilinogen deaminase have been determined. The Michaelis constant with porphobilinogen as substrate is unchanged in the presence of porphobilinogen α-carboxylic acid.

4. Uroporphyrinogen decarboxylase acts on all four position-isomers of uroporphyrinogen and is unstable in the absence of sulphydryl compounds.

5. *Rhodopseudomonas spheroides*, chick blood, yeast, and pig liver contain a heat-stable factor which stimulates coproporphyrin formation from porphobilinogen.

The authors gratefully acknowledge the technical assistance of Mr Alan Strauss and Miss Sylvia Tebb, and thank Professor C. Rimington, F.R.S., for his encouragement and support of this work.

REFERENCES

Bogorad, L. (1957). *Plant. Physiol.* Suppl. **32**, xli.
Bogorad, L. (1958*a*). *J. biol. Chem.* **233**, 501.
Bogorad, L. (1958*b*). *J. biol. Chem.* **233**, 510.
Bogorad, L. (1958*c*) *J. biol. Chem.* **233**, 516.
Bogorad, L. & Granick, S. (1953). *Proc. nat. Acad. Sci., Wash.*, **39**, 1176.
Booij, H. L. & Rimington, C. (1957). *Biochem. J.* **65**, 4P.
Brockman, P. E. & Gray, C. H. (1953). *Biochem. J.* **54**, 22.
Carpenter, A. T. & Scott, J. J. (1959). *Biochem. J.* **71**, 325.
Cooper, R. (1956). *Biochem. J.* **63**, 25P.
Eriksen, L. (1958). *Scand. J. clin. Lab. Invest.* **10**, 319.
Falk, J. E. & Benson, A. (1953). *Biochem. J.* **55**, 101.
Falk, J. E., Dresel, E. I. B., Benson, A. & Knight, B. C. (1956). *Biochem. J.* **63**, 87.
Granick, S. & Mauzerall, D. (1958). *J. biol. Chem.* **232**, 1119.
Grant, G. H., Rowe, D. S. & Stanworth, D. R. (1958). *Biochem. J.* **69**, 13P.
Heath, H. & Hoare, D. S. (1959*a*). *Biochem. J.* **71**, 5P.
Heath, H. & Hoare, D. S. (1959*b*). *Biochem. J.* **72**, 14.
Hoare, D. S. & Heath, H. (1958). *Nature, Lond.*, **181**, 1592.
Lockwood, W. H. & Rimington, C. (1957). *Biochem. J.* **67**, 8P.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Mauzerall, D. & Granick, S. (1958). *J. biol. Chem.* **232**, 1141.
Nicholas, R. E. H. (1951). *Biochem. J.* **48**, 309.
Rimington, C. & Sveinsson, S. L. (1950). *Scand. J. clin. Lab. Invest.* **2**, 209.
Vernon, L. P. & Kamen, M. D. (1954). *J. biol. Chem.* **211**, 643.

The Chemistry of Xanthine Oxidase

6. VARIATIONS IN STABILITY AND THE PRESENCE OF AN INHIBITOR IN CERTAIN PREPARATIONS*

By R. C. BRAY
Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, London, S.W. 3

(*Received* 8 *June* 1959)

It has been reported previously that the stability of xanthine oxidase samples varies from one preparation to another (Bergel & Bray, 1956, 1959). This paper reports results of a comparison of the properties of two preparations of the enzyme, one of which proved to be exceptionally unstable, whereas the other was rather stable. Kinetic data indicate that the stable preparation contained a competitive inhibitor of xanthine oxidase, the presence of which could account for the stability differences between the two samples.

MATERIALS AND METHODS

Xanthine oxidase solutions. These were prepared by methods described previously (cf. Bergel & Bray, 1959). Most of the experiments were carried out on two preparations, namely LXO–70 and LXO–116. The former was purified by the method of Avis, Bergel & Bray (1955) to stage M5 and had an activity/E_{450} ratio of about 40; the latter was prepared by ammonium sulphate treatment (Haddow, de Lamirande, Bergel, Bray & Gilbert, 1958) and had an activity/E_{450} ratio which fell from 50 to about 10 during the period over which experiments were carried out. The samples both had E_{280}/E_{450} ratios of around 10. They were stored at 0–5° as solutions containing about 1% of xanthine oxidase (calculated from E_{450}). Copper analyses were carried out by the method of Eden & Green (1940).

Chemicals and buffer solutions. These were generally the same as those used previously (Bergel & Bray, 1959). Catalase was obtained from General Biochemicals Inc., Chagrin Falls, Ohio, U.S.A., and activity measurements indicated a purity of the order of 5%; soy-bean trypsin inhibitor was obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Pyrophosphate buffer for the enzyme assays was prepared by mixing $Na_4P_2O_7$ and $Na_2H_2P_2O_7$; M-phosphate buffer, pH 5·8, contained 0·2M-Na_2HPO_4 and 0·8M-KH_2PO_4; the other phosphate buffers were diluted from this solution.

Enzyme assays and stability experiments. The methods employed were similar to those of Bergel & Bray (1959). However, the xanthine oxidase assays, apart from those in connexion with the stability tests, were carried out at 305 mμ, as solutions of xanthine have a lower absorption at this wavelength than at the one normally used, i.e. 295 mμ. At 305 mμ the value of $\Delta\epsilon$ for the conversion of xanthine into uric acid is about half of the corresponding figure at 295 mμ (cf. Avis, Bergel & Bray, 1956). In some cases it was found convenient to carry out assays in 0·5 or 4·0 cm.

* Part 5: Bray, Malmström & Vänngård (1959).

Table 1. *Stability of xanthine oxidase preparations LXO–70 and LXO–116*

Losses of activity (with xanthine as substrate and oxygen as electron acceptor) of the two samples were compared in a series of parallel experiments at a concentration of approx. 0·1% xanthine oxidase, under the conditions indicated. The buffer tris maleate is 2-amino-2-hydroxymethylpropane-1:3-diol–maleic acid (Bergel & Bray, 1959).

Temp.	Duration	pH	Buffer	Additives	Activity remaining (%) LXO–70	LXO–116
0–5°	19 days	7·0	0·08 M-Tris maleate	—	94	69
0	24 hr.	7·0	0·08 M-Tris maleate	0·001 M-Ascorbic acid	92	86
56	3 hr.	5·8	0·8 M-Phosphate	0·03 M-Salicylate 0·01 M-EDTA	74	56
56	3 hr.	6·2	0·01 M-Phosphate	0·03 M-Salicylate 0·5 mg. of catalase/ml.	70	33
56	3 hr.	6·2	0·01 M-Phosphate*	0·03 M-Salicylate	47	19
					Mixture†: 40	

* Xanthine oxidase solutions were dialysed for 18 hr. in separate bags against the same buffer solution (approx. 1000 vol.) at 0–5° before the experiment; activities are expressed as percentages of that after dialysis.

† Mixed after dialysis; total xanthine oxidase concentration was as for experiments on separate batches.

spectrophotometer cells in place of the usual 1·0 cm. type. For assays at low substrate concentrations it was important to start taking readings as soon as possible after starting the reaction; when combined enzyme samples were assayed, the xanthine oxidase solutions were therefore mixed before they were added to the buffered xanthine solution.

RESULTS

During ordinary storage in the refrigerator, xanthine oxidase preparation LXO–116 (in 0·01 M-phosphate buffer, pH 6·2, with 1 mM-salicylate) lost about 50% of its activity during 1 month, whereas with preparation LXO–70 (0·1 M-phosphate, pH 6·1, without salicylate) activity decreased at only about 5% per month. Data from a number of stability tests in which LXO–70 and LXO–116 were compared in parallel experiments are reported in Table 1; the latter sample lost activity at a higher rate than the former under all the conditions tried.

In an attempt to find differences other than of stability between the two xanthine oxidase samples, they were analysed for copper, this being a possible inactivating contaminant (Bergel & Bray, 1959). Both samples contained significant quantities of the metal, but the amounts were roughly equal, and corresponded to about 0·5 g.atom of copper/mole of xanthine oxidase in each case.

The effects of substrate and of enzyme concentration on the enzymic activity with xanthine as substrate and oxygen as electron acceptor were then studied (Fig. 1). It was found that the preparations differed markedly from one another, LXO–70 showing, at high enzyme concentrations, a decrease in specific activity, an increase in the xanthine concentration required for maximum activity and a broadening of the peak of the curve; these phenomena were not found with LXO–116. In dilute xanthine oxidase solution, LXO–70 gave maximum

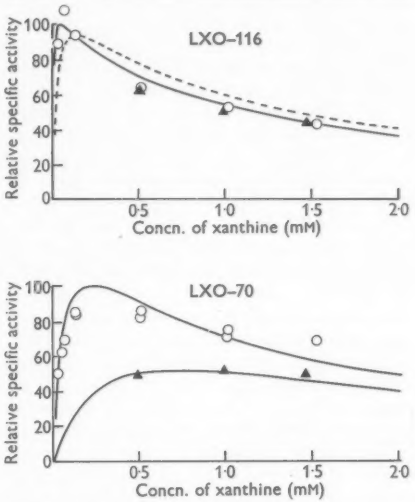


Fig. 1. Effect of xanthine concentration on xanthine oxidase activity at 24° in 0·05 M-pyrophosphate buffer, pH 8·2. Relative specific activity = $k(\Delta E/\text{min.})$ (vol. in cell)/(light path) (vol. of xanthine oxidase), where k is an arbitrary constant; different values of k were assumed for the different xanthine oxidase samples used in this experiment, but not for different dilutions of the same sample. Overall dilutions of xanthine oxidase were: ▲, 1 in 26; ○, 1 in 260. All the curves are theoretical ones calculated by means of equation (9) of Hofstee (1955). The broken line was calculated with Hofstee's constants: K_s 2×10^{-5}, K_{ss} 10^{-3}, taking V_m 122 and assuming no inhibitor. The continuous lines were calculated with K_s 10^{-6} and K_{ss} 10^{-3}, assuming no inhibitor for LXO–116 and $[I]/K_i$ 50 and 500 for the upper and lower curves respectively for LXO–70; V_m 107 for LXO–116 and 150 for LXO–70.

activity at about 0·2 mM-xanthine, whereas for LXO–116 the corresponding value was about 0·05 mM.

Further data showing the decrease of the specific activity of LXO–70 at high enzyme concentrations with a low xanthine concentration are presented in Fig. 2. This figure also shows that addition of increasing amounts of LXO–70 to a fixed amount of LXO–116 resulted in a progressive decrease in the activity of the latter sample.

Experiments similar to those reported in Fig. 1 were carried out on a number of other xanthine oxidase samples at various stages of purification, including a buttermilk sample. In all cases the forms of the curves obtained were intermediate in shape between those given by LXO–70 and LXO–116. LXO–70 had been dialysed thoroughly about 9 months before the experiments reported above, but re-dialysis shortly before assay resulted in a sharpening in the peak of the curve, though the shape remained intermediate between its original form and that given by LXO–116. Dialysis of LXO–116 did not change the shape of its curve.

When samples of either LXO–70 or LXO–116 (without dialysis) were denatured by heating at 100° in 0·05 M-pyrophosphate buffer, pH 8·2, and the supernatants were added to portions of LXO–116, some reduction of the activity of the latter sample occurred, the xanthine oxidase assay being performed at a low xanthine concentration.

The addition of trypsin inhibitor (final concentration approx. 0·1 mM) to a solution of xanthine oxidase did not result in any decrease in the activity of the enzyme.

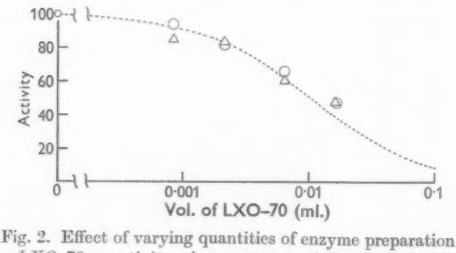


Fig. 2. Effect of varying quantities of enzyme preparation LXO–70 on activity. Assays were carried out at 24° with 0·05 mM-xanthine in a total volume of 2·5 ml. with 0·05 M-pyrophosphate buffer, pH 8·2. △, Relative specific activity of LXO–70 alone (calc. as in Fig. 1); ○, activity of a fixed amount of LXO–116 in the presence of added LXO–70, calculated as

$$\frac{\text{(Activity of mixture)} - \text{(activity of LXO–70 alone)}}{\text{(Activity of LXO–116 alone)}} \times 100.$$

The theoretical curve through the points was calculated by the use of equation (11) of Hofstee (1955), with K_s 10^{-6}, K_{ss} 10^{-3} and v 100, and taking values of $[I]/K_i$ appropriate to the dilution of LXO–70, on the same basis as for Fig. 1.

DISCUSSION

Kinetic experiments. The kinetic data are consistent with the presence of a competitive inhibitor of xanthine oxidase in LXO–70. Hofstee (1955) has studied the effects of substrate concentration on the activity of xanthine oxidase in the presence of competitive inhibitors; he could explain his results by equations obtained assuming the presence of two active sites in the xanthine oxidase molecule. The first of these was the primary substrate site; his inhibitors competed with xanthine for it and the Michaelis constant for xanthine at this site was K_s 2×10^{-5}. Xanthine also combined with the second site, which may be the one normally used by the electron acceptor; the dissociation constant (K_{ss}) was 10^{-3} and this combination was the cause of the inhibition which occurs at high substrate concentrations.

The broken line in Fig. 1 was calculated for LXO–116 by using Hofstee's equation for xanthine oxidase in the absence of an inhibitor and taking his values for the constants; a good fit with the experimental data could not be obtained, whatever value of V_m was assumed. Other values of the constants were therefore tried, and it was found that if K_{ss} was left unchanged and K_s was taken as 10^{-6}, a much better fit was achieved. Accepting for the time being these latter values as the true constants for inhibitor-free xanthine oxidase, it was then assumed that an inhibitor was present in LXO–70. Hofstee's equation being used once again, various values of $[I]/K_i$ were tried until the best fit of the theoretical curves to the experimental data for this enzyme sample was obtained. As shown in Fig. 1, a satisfactory fit at both the xanthine oxidase concentrations at which assays were carried out was achieved. The value $[I]/K_i$ $1{\cdot}3 \times 10^4$ for undiluted LXO–70, which was used in the calculations for Fig. 1, also fits further data on the activity of various concentrations of this xanthine oxidase batch at a fixed xanthine concentration (Fig. 2). Fig. 2 also shows that, on mixing the two enzyme samples, LXO–116 was inhibited to about the extent required on the basis of the above-mentioned theory.

The presence of an inhibitor in LXO–70 but not in LXO–116 thus gives, in so far as the experimental points in Figs. 1 and 2 fall on the theoretical curves, a satisfactory explanation of the results of the kinetic experiments. The discrepancy between the present value of K_s, i.e. 10^{-6}, and that given by Hofstee (1955) could be explained by assuming that his enzyme contained an inhibitor similar to the one present in LXO–70. If his assay solutions had $[I]/K_i$ 19, the value of the 'apparent' Michaelis constant, K_s $(1+[I]/K_i)$, would be equal to his value of 2×10^{-5} and his results would fall into line

with those presented above. Figures in the literature for the K_s value of xanthine oxidase vary widely (cf. Fridovich & Handler, 1958*b*), but the lowest recorded value of K_s $0{\cdot}9 \times 10^{-6}$ (Lowry, Bessey & Crawford, 1949) is in good agreement with the present work. It therefore seems likely that the higher values recorded by a number of workers may have been an indication of the presence of inhibitors in their preparations. Two other points in the literature which could be explained on a similar basis are the variations of K_s from one xanthine oxidase preparation to another, reported by Booth (1938), and a flattening of the peak of maximum activity on the xanthine-activity graphs at high xanthine oxidase concentrations, which was observed by Dixon & Thurlow (1924).

Stability experiments. The differences of stability between LXO–70 and LXO–116, which remained in the presence of ethylenediaminetetra-acetic acid (EDTA) or of ascorbic acid, and the similarity of their copper contents suggested that trace-metal impurities were not responsible for the stability differences (cf. Bergel & Bray, 1959). Similarly, as catalase did not affect the relative stabilities the differences could not be due to hydrogen peroxide inactivation. The fact that mixing the batches resulted in a stability slightly nearer to that of LXO–70 than to LXO–116 (Table 1) suggested that the former might contain a protecting agent of some kind. Bergel & Bray (1959) showed that competitive inhibitors stabilized xanthine oxidase and, as the kinetic results discussed above showed that LXO–70 contained an inhibitor, it would have been reasonable to suppose that this was the protecting agent responsible for the stability differences. However, two difficulties remained to be explained before this hypothesis could be accepted. As shown in Table 1, the addition of an excess of salicylate, which is a known xanthine oxidase inhibitor–stabilizer, did not bring the stability of LXO–116 up to that of LXO–70. It is therefore necessary to postulate that the complex between the unknown inhibitor present in LXO–70 and the xanthine oxidase molecule is less sensitive to inactivation than is the salicylate–xanthine oxidase complex, which is in turn, of course, more stable than the free xanthine oxidase molecule. This assumption seems to be a plausible one as the inhibitor may be quite different chemically from salicylate and might combine with the active centre of xanthine oxidase in a different manner. Questions about the dialysibility of the inhibitor are discussed below.

Nature of the inhibitor. The kinetic results give a value for $[I]/K_i$ for LXO–70, but not for K_i. However, it is possible to arrive at some idea of the magnitude of this constant by making an estimate of the value of $[I]$. The inhibitor is unlikely to be present in LXO–70 at above, say, 0·01 M, and a lower limit for it is set by the concentration of active xanthine oxidase in the sample, which is about 10 μM. Hence the value of K_i probably lies between about 10^{-6} and 10^{-9}, and the inhibitor is thus quite a potent one, which would not be expected to be removed completely from the solution by ordinary dialysis. Although, as the kinetic results indicated, the concentration of inhibitor was substantially reduced by dialysis, it was apparently not completely eliminated; the stability experiments confirmed that a stabilizing factor remained in LXO–70 after dialysis. Thus all the experimental data could be explained satisfactorily on the assumption that the inhibitor and the stabilizer of LXO–70 were identical.

There is very little information about the chemical nature or the source of the inhibitor, but it seems that it must have been present in substantial amounts in LXO–70, and it may be significant that this sample had been subjected to prolonged storage at 5°. It was also apparently present in smaller quantities in a number of other xanthine oxidase preparations, including a buttermilk sample, whereas it was absent from LXO–116. (The reason for this absence is not clear, but it does not seem to have anything to do with the method used for the preparation of the sample, since other samples prepared in the same manner were much more stable.) Somewhat unexpectedly, however, an inhibitor was found in solutions obtained from LXO–116 after denaturation at 100°. It is possible that we may be concerned with more than one xanthine oxidase inhibitor, and it is perhaps tempting to speculate, in the absence of any direct evidence, that they may be peptides analogous to the well-known trypsin inhibitors (cf. Laskowski & Laskowski, 1954); however, their dialysibility suggests a somewhat lower molecular weight. (Soybean trypsin inhibitor, which is capable of interacting with a number of proteolytic enzymes in addition to trypsin, was without inhibitory effect on xanthine oxidase.) If, as seems probable from these considerations, denaturation of the xanthine oxidase molecule can lead to the formation of an inhibitor, this could explain the break in the inactivation curves of xanthine oxidase at 56° observed by Bergel & Bray (1959). The rate of loss of activity would decrease as the inhibitor concentration increased and became unimolecular when sufficient of it was present to 'saturate' the remaining xanthine oxidase. Variations in the inhibitor concentration may account for many of the batch variations in xanthine oxidase stability which are not explained by previous work (Bergel & Bray, 1959).

It seems unlikely that any of the inhibitory effects reported in this paper could be due to the presence of 'inactive' xanthine oxidase molecules. Though not catalytically active, these might well

still be capable of substrate binding (cf. hypoxanthine binding by cyanide-treated xanthine oxidase, Fridovich & Handler, 1958*a*). However, in this case inhibition would be due to substrate–inhibitor rather than enzyme–inhibitor combination; this type of inhibition is important only when a sufficient number of inhibitor molecules are present to bind an appreciable proportion of the substrate molecules (Friedenwald & Maengwyn-Davies, 1954), and as the total xanthine oxidase concentration calculated from the E_{450} value did not exceed 1 μM in any of the assays, this type of inhibition may clearly be neglected in this work.

SUMMARY

1. The stabilities of two xanthine oxidase preparations have been compared under a variety of conditions; one of these preparations was found to be consistently more stable than the other.

2. Kinetic experiments indicated that the more stable sample contained a xanthine oxidase inhibitor; the presence of this could account for the observed stability differences.

3. Evidence bearing on the occurrence, formation and nature of the inhibitor are discussed briefly, and it is suggested that variations in its concentration may be responsible for certain discrepancies and anomalies in the properties of xanthine oxidase recorded in the literature.

The author thanks Professor F. Bergel, F.R.S., for his interest in this work and for many helpful discussions. The xanthine oxidase samples were prepared by Mr P. G. Avis and Mr D. A. Gilbert, and Miss A. M. Scott rendered valuable technical assistance. The work has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

REFERENCES

Avis, P. G., Bergel, F. & Bray, R. C. (1955). *J. chem. Soc.* p. 1100.
Avis, P. G., Bergel, F. & Bray, R. C. (1956). *J. chem. Soc.* p. 1219.
Bergel, F. & Bray, R. C. (1956). *Nature, Lond.*, **178**, 88.
Bergel, F. & Bray, R. C. (1959). *Biochem. J.* **73**, 182.
Booth, V. H. (1938). *Biochem. J.* **32**, 503.
Bray, R. C., Malmström, B. G. & Vänngård, T. (1959). *Biochem. J.* **73**, 193.
Dixon, M. & Thurlow, S. (1924). *Biochem. J.* **18**, 976.
Eden, A. & Green, H. H. (1940). *Biochem. J.* **34**, 1202.
Fridovich, I. & Handler, P. (1958*a*). *J. biol. Chem.* **231**, 899.
Fridovich, I. & Handler, P. (1958*b*). *J. biol. Chem.* **233**, 1578.
Friedenwald, J. S. & Maengwyn-Davies, G. D. (1954). In *Mechanism of Enzyme Action*, p. 154. Ed. by McElroy, W. D. & Glass, B. Baltimore: Johns Hopkins University Press.
Haddow, A., de Lamirande, G., Bergel, F., Bray, R. C. & Gilbert, D. A. (1958). *Nature, Lond.*, **182**, 1144.
Hofstee, B. H. J. (1955). *J. biol. Chem.* **216**, 235.
Laskowski, M. & Laskowski, M. (1954). *Advanc. Protein Chem.* **9**, 203.
Lowry, O. H., Bessey, O. A. & Crawford, E. J. (1949). *J. biol. Chem.* **180**, 399.

Arteriovenous $^{14}CO_2$ Differences and the Pentose Cycle in the Cow's Udder

By H. G. WOOD, R. GILLESPIE, R. G. HANSEN,* W. A. WOOD* and H. J. HARDENBROOK
Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland, Ohio, and the Department of Dairy Science and College of Veterinary Medicine, University of Illinois, Urbana, Illinois, U.S.A.

(*Received* 23 *March* 1959)

There is a large body of evidence that the pentose cycle occurs in the mammary gland. This evidence is based: (1) on studies of the conversion of different types of labelled glucose into CO_2 (Abraham, Hirsch & Chaikoff, 1954; Glock, McLean & Whitehead, 1956; Duncombe & Glascock, 1956; Abraham, Cady & Chaikoff, 1957; Black, Kleiber, Butterworth, Brubacher & Kaneko, 1957); (2) on measurement of glucose 6-phosphate and 6-phosphogluconate dehydrogenases (Glock & McLean, 1953, 1954); (3) on the detection of some of the intermediate compounds of the pentose cycle in mammary tissues (Peeters & Debackere, 1956); and (4) on the determination of the distribution of ^{14}C in the 6 carbons of the glucose and galactose moieties of lactose (Schambye, Wood & Kleiber, 1957; Wood, Gillespie, Joffe, Hansen & Hardenbrook, 1958*a*; Wood, Joffe, Gillespie, Hansen & Hardenbrook, 1958*b*). However, Duncombe & Glascock (1956) have indicated that the direct oxidative pathway may not be as important in the udder of ruminants (sheep) as in the rat mammary

* Present address: Department of Agricultural Chemistry, Michigan State University, East Lansing, Michigan.

gland. Also Glock *et al.* (1956) state that in the mammary gland of sheep there was not a large increase in glucose 6-phosphate and 6-phosphogluconate dehydrogenase during lactation such as was observed in rats.

The investigations of the pentose cycle in mammary gland by means of labelled glucose have for the most part been done *in vitro* with slices. The investigation of Black *et al.* (1957) is an exception. These authors injected [1-^{14}C]- and [6-^{14}C]-glucose intravenously into lactating cows and found 1·6–3·6 times as much ^{14}C in the respiratory CO_2 with [1-^{14}C]- as with [6-^{14}C]-glucose. They also found more ^{14}C from [6-^{14}C]- than from [1-^{14}C]-glucose in the alanine and serine of casein and in the glycerol of fat. Both results indicate a pentose cycle and they conclude that the cycle plays a major role in glucose metabolism in the lactating cow.

Previous studies have shown that it is possible to investigate the metabolism of the udder in the intact cow (Wood, Siu & Schambye, 1957; Wood *et al.* 1958*a*, *b*) by injection of ^{14}C compounds directly into a pudic artery which supplies blood to only one side of the udder. A high specific activity of ^{14}C was observed in the milk from the injected side, which reflects the metabolism of the compound directly in the udder, and a lower activity was observed in the milk from the uninjected side which reflects the metabolism of the compound in the whole animal. In the present study the metabolism of the udder has been investigated by injecting labelled glucose into the arterial supply and then determining the arteriovenous difference in specific activity of CO_2 of the blood supply of the udder. The C-1 of glucose is oxidized in the udder to CO_2 far more rapidly than is C-6. [1-^{13}C]- or [1-^{14}C]-acetate was also included in the study and it is oxidized to CO_2 as rapidly as is the C-1 of glucose.

METHODS

The arterial blood supply of the cow's udder flows via the posterior aorta to the right and left external iliac arteries and then to the corresponding right and left pudic arteries. The major return of blood from the udder is through the right and left subcutaneous abdominal veins and the right and left pudic veins (Espe, 1948). There are collateral connexions in the venous circulation of the two sides of the udder, but relatively few in the arterial circulation (Turner, 1952). Arterial blood usually was obtained from either the posterior aorta or pudic artery, but in one experiment the blood of the jugular vein was taken as representative of arterial blood. The ^{14}C of the CO_2 leaving the udder was determined in blood from the subcutaneous abdominal vein. The arteriovenous difference in the specific activity of the CO_2 of the blood of the udder is considered to be proportional to the rate of oxidation of the labelled carbon of the compound tested.

A Holstein cow weighing about 750 kg. and producing about 22 l. of milk per day was used in the experiments of Tables 1–3. She had been used previously in experiments by Wood *et al.* (1958*a*, *b*). For the experiment of Table 4 a Holstein cow was used which weighed about 500 kg. and produced about 18 l. of milk/day. Local anaesthesia of the left flank of the cow was produced by injecting procaine into the left epidural space of the lumbar spinal column (St Clair & Hardenbrook, 1956; Wood *et al.* 1958*b*). With the cow in a normal standing position an incision was made through the abdominal wall, and the posterior aorta and left pudic artery were located by palpation. The labelled compound was infused into the pudic artery or posterior aorta by using a constant-speed mechanical syringe attached with Tygon tubing (Arthur H. Thomas, Philadelphia) to a hypodermic needle. Blood was drawn at as nearly the same rate as possible from the selected vessels. It required about 30 sec. to draw the blood and the time given is the mid-point of the collection.

The CO_2 was liberated from the blood samples in an all-glass apparatus with ground-glass joints consisting of a 500 ml. flask fitted with a funnel and connected by a large-bore tube to a 50 ml. flask. The 500 ml. flask contained 100 ml. of H_2O, 5 ml. of 5N-H_2SO_4 and 1 ml. of octyl alcohol. A volume (3 ml.) of 2·5N-NaOH was in the 50 ml. flask and 2 ml. of mineral oil and 3 ml. of 3·3% sodium citrate were in the funnel. The assembled equipment was evacuated before the experiment. The blood was introduced in the funnel under the mineral oil and then was rapidly admitted to the 500 ml. flask. The 500 ml. flask then was shaken in a hot-water bath for 5 min. while the 50 ml. flask was cooled in ice. The ^{14}C and ^{13}C of the CO_2 were determined with a gas-phase proportional counter (Bernstein & Ballentine, 1950) and a double-beam mass spectrometer (Nier, 1947) respectively.

In some of the experiments the acetate or glucose, or both, were isolated from the blood. The acidified blood from the 500 ml. flask was neutralized to pH 7 with NaOH solution and then deproteinized with 40 ml. of 5% $ZnSO_4$ and 40 ml. of 0·3N-$Ba(OH)_2$. The filtrate from this mixture was made just alkaline to phenolphthalein and was then freeze-dried. The dry powder was dissolved in water, acidified to a Congo red end-point and extracted continuously with ether into water. The combined ether and water of the extract was steam-distilled, the volatile acids were titrated and the solution was evaporated to dryness. The volatile acids were then chromatographed on a Celite column (Swim & Krampitz, 1954) to obtain the acetic acid, which was titrated, diluted with carrier acetate, and then oxidized to CO_2 (Van Slyke & Folley, 1950). The ^{13}C content of the CO_2 was determined and the total excess of ^{13}C was calculated for the carboxyl carbon of the acetate.

The residue of the ether extraction was deionized with Amberlite resin, the reducing sugar was determined (Somogyi, 1945; Nelson, 1944), carrier glucose was added and the phenylosazone was prepared and recrystallized (Baker, Shreeve, Shipley, Incefy & Miller, 1954). The osazone was oxidized to CO_2 (Van Slyke & Folley, 1940), its ^{14}C content was determined and the specific activity of the osazone was calculated.

RESULTS AND DISCUSSION

Comparison of oxidation of [1-^{14}C]- *and* [6-^{14}C]-*glucose to* CO_2. In the first experiment 100 μc of labelled glucose was injected into the left pudic

artery over a period of 15 sec. and then blood was collected at intervals from the left subcutaneous abdominal and jugular veins. The metabolism of [1-^{14}C]- and [6-^{14}C]-glucose was tested alternately (Table 1). The CO_2 of the blood of the jugular vein was assumed for purposes of this experiment to be comparable with that of the arterial blood supplying the udder. This assumption was valid, as illustrated in the experiment of Table 3, in which blood from both the posterior aorta and jugular vein was collected.

There was an immediate rise in the specific activity of the CO_2 of the subcutaneous abdominal vein above that of the jugular vein when [1-^{14}C]-glucose was injected (tests *A* and *C*). The highest difference was observed at the time of the first collection, 2 min. after the injection. The values for the two tests were not the same, however, test *A* giving an arteriovenous difference of 12·0 counts/min./μmole of CO_2, and test *C* giving 3·8. It seems likely that the principal cause of disagreement was the timing of the injection and of the collection of the blood. Apparently the specific activity of the CO_2 rose and fell rapidly and was falling rapidly at 2 min. With [6-^{14}C]glucose, test *B*, there was only a small arteriovenous difference in the specific activity of the CO_2 of the blood and in test *D* the arteriovenous difference was actually reversed, i.e. it was slightly higher in the jugular vein. The results clearly show that [1-^{14}C]glucose gives rise to CO_2 more rapidly than does [6-^{14}C]glucose and thus indicates that the pentose cycle occurs in the udder. These experiments, therefore, supplement those of Black *et al.* (1957), which were based on the composite metabolism of the whole cow.

The changes in the specific activity of the CO_2 of the blood in the subcutaneous abdominal vein were very different from those found in the respiratory CO_2 by Black *et al.* (1957). They found that the maximum specific activity was reached in about 30 min. and then the ^{14}C activity fell slowly. This delayed maximum and prolonged high activity apparently occurred because the labelled glucose mixed with the large body pool of glucose and after the initial equilibration the specific activity of the glucose did not change rapidly (Black *et al.* 1957; Kleiber *et al.* 1955). In addition, the bicarbonate and CO_2 pools of the body are large and consequently considerable time is required for these pools to reach their maximum specific activity. In contrast, injection via the pudic artery rapidly raises the specific activity of the glucose in the udder, but the specific activity of the glucose in the whole body pools remains relatively low. However, as soon as the injection is stopped the ^{14}C activity of the glucose in the udder drops because glucose with low activity now enters the udder. Consequently there was a rapid rise and fall in the specific activity of the blood CO_2 in the subcutaneous abdominal vein. However, the changes in specific activity of the CO_2 of the blood from the jugular vein were similar to those observed by Black *et al.* (1957) in the respiratory CO_2, i.e. reaching a maximum at about 26 min. and then remaining constant. This similarity is to be expected, since both the blood CO_2 of the jugular vein and respiratory CO_2 reflect the changes in the pools of the entire body.

Equilibration between blood and tissue bicarbonate in the udder. The fact that the specific activity of

Table 1. *Arteriovenous difference in specific activity of* CO_2 *of the blood supply of the udder after injecting* [1-^{14}C]- *or* [6-^{14}C]-*glucose in the left pudic artery of a cow*

Aq. 0·09% NaCl containing 100 μc of labelled glucose (National Bureau of Standards) (5 ml.) was injected into the left pudic artery in approximately 15 sec. Blood (20 ml.) was withdrawn at the indicated times simultaneously from the left subcutaneous abdominal vein and the jugular vein, and the CO_2 was collected from the blood. The injections of labelled sugars were at 3.12, 3.58, 4.49 and 5.40 p.m. After completion of test *B*, 50 units of oxytocin were injected in the jugular vein and the cow was milked; 12 l. was obtained.

Values are specific activities expressed as counts/min./μmole of CO_2. The time is that after completion of the injection of the labelled glucose.

	Test *A* [1-^{14}C]Glucose					Test *B* [6-^{14}C]Glucose				
Time (min.)	2	6	14	26	42	2	6	14	26	42
Subcutaneous abdominal vein	12·2	3·08	1·62	1·21	1·18	1·29	1·34	1·10	1·13	1·13
Jugular vein	0·2	0·99	1·13	1·24	1·10	1·06	1·16	1·31	1·13	1·29
Arteriovenous difference	12·0	2·09	0·49	−0·04	0·08	0·23	0·18	−0·21	0·00	−0·16
	Test *C* [1-^{14}C]Glucose					Test *D* [6-^{14}C]Glucose				
Time (min.)	2	6	14	26	42	2	6	14	26	42
Subcutaneous abdominal vein	5·32	—	2·26	2·29	2·19	2·18	2·45	2·48	2·66	2·82
Jugular vein	1·52	1·94	2·31	2·74	2·76	2·78	2·89	2·87	2·65	3·14
Arteriovenous difference	3·80	—	−0·05	−0·45	−0·57	−0·60	−0·44	−0·39	−0·01	−0·32

the CO_2 was sometimes higher in the jugular vein than in the subcutaneous abdominal vein (Table 1) was of interest and was not expected. It seemed likely that this dilution of $^{14}CO_2$ of the blood was due in part to an equilibration with unlabelled bicarbonate of the different pools in the udder, including that of the milk. The experiment in Table 2 was done to obtain information on the rate of this equilibration. This information was essential for interpretation of data from the experiments with labelled glucose and acetate. A solution of $NaH^{14}CO_3$ was injected at constant rate into the posterior aorta and blood samples were obtained during the course of the injection from the left pudic artery and the left subcutaneous abdominal vein. The amount of CO_2 in the blood and its specific activity were determined and from these values the total ^{14}C in the blood was calculated. The results are shown in Table 2.

The specific activity of the CO_2 of the blood decreased greatly as it passed through the udder. At 8 min. the decrease was 32 %. The increase in the arteriovenous CO_2 was from 533 to 584 μmoles/20 ml. of blood and therefore was not enough to account for the dilution of $^{14}CO_2$ which occurred in the blood. Clearly this decrease in specific activity was in large part due to equilibration with unlabelled bicarbonate of the udder. This fact is apparent since the total counts/min. as CO_2 in the blood decreased as it passed through the udder, while there was no net uptake of CO_2 from the blood. The decrease in ^{14}C activity varied from 43 % at 2 min. to 26 % at 8 min. The results indicate clearly the complications in experiments involving arteriovenous differences of CO_2 formed from labelled compounds. It should theoretically be possible by use of the isotope-dilution technique to determine the total CO_2 produced from unlabelled compounds in the metabolism of the udder or, conversely, to determine the amount of CO_2 produced from a labelled compound. However, this would be difficult because it would be necessary to know the size of the bicarbonate pool of the udder and blood and the rate of equilibration of the bicarbonate of the blood and udder. During a period of time sufficient for complete equilibration to occur, compounds other than the one injected would acquire ^{14}C activity owing to metabolism in the udder or in other organs of the body, and these compounds would in turn contribute to the $^{14}CO_2$.

Comparison of oxidation of C-1 *of glucose and* C-1 *of acetate to carbon dioxide.* Baxter, Kleiber & Black (1955) have estimated that the lactating cow derives only 10 % of its CO_2 from pathways involving oxidation of glucose and that glucose is less important in the energy metabolism of the cow than it is in the metabolism of dogs and rats. This is in accord with the fact that most of the non-nitrogenous material absorbed from the digestive tract of ruminants is in the form of volatile fatty acids (Barcroft, McAnally & Phillipson, 1944). It is possible, however, that the metabolism of glucose may play a proportionally greater part in the udder than in the whole animal, since the pentose cycle may serve as a major source of reduced triphosphopyridine nucleotide for the synthesis of fat in the mammary gland (Seubert, Greult & Lynen, 1957; Lachance, Popják & de Waard, 1958). It therefore was of interest to compare the oxidation of the C-1 of glucose with the C-1 of acetate. For this purpose [1-^{14}C]glucose and [1-^{13}C]acetate were injected into the left pudic artery, and the glucose and acetate from the blood of the left subcutaneous abdominal vein were isolated and their ^{14}C and ^{13}C contents

Table 2. *Arteriovenous difference in the specific activity of the bicarbonate of blood during its passage through the udder*

Bicarbonate solution (2·5 ml.) containing about $9{\cdot}8 \times 10^6$ counts/min. was injected per min. into the posterior aorta. The collection of blood from the left pudic artery and the left subcutaneous abdominal vein was started 2 min. after the beginning of the injection. The injection of $NaH^{14}CO_3$ was continuous until all samples of blood were collected.

An average of 533 μmoles of CO_2 was found in 20 ml. of blood of the pudic artery and 584 μmoles in 20 ml. of blood of the subcutaneous abdominal vein. The total ^{14}C was calculated from these values and the corresponding specific activities of the CO_2 (e.g. $533 \times 26{\cdot}5 = 14120$ or $14{\cdot}12 \times 10^3$).

		Time (min.)			
		2	4	6	8
Specific activity of CO_2 of blood (counts/min./μmole)	Pudic artery	26·5	—	30·6	29·6
	Subcutaneous abdominal vein	13·8	15·3	19·0	20·1
Arteriovenous difference	—	12·7	—	11·6	9·5
Decrease in specific activity (%)	—	48	—	38	32
$10^{-3} \times$Total ^{14}C in CO_2 of 20 ml. of blood (counts/min.)	Pudic artery	14·12	—	16·32	15·80
	Subcutaneous abdominal vein	8·06	8·94	11·10	11·72
Decrease in total ^{14}C	—	6·06	—	5·22	4·08
Decrease in total ^{14}C (%)	—	43	—	32	26

determined. The arteriovenous ^{14}C and ^{13}C differences of the blood CO_2 were calculated. The relative rates of oxidation of the [1-^{14}C]glucose and [1-^{13}C]-acetate to CO_2 were to be compared on the basis of the following ratios: (1) specific activity of blood CO_2 divided by specific activity of blood glucose; (2) percentage excess of ^{13}C in CO_2 divided by percentage excess of ^{13}C in the acetate. Since the $^{13}CO_2$ and $^{14}CO_2$ presumably would undergo dilution in the same pools, complete equilibration of the blood bicarbonate and tissue bicarbonate would not be necessary for purposes of this comparison.

The results are shown in Table 3. After injection of labelled glucose and acetate there was no detectable difference between the ^{14}C activity of the CO_2 of the jugular vein and that of the posterior aorta, apparently because of the low specific activity of the blood glucose in the general circulation. On the injected side of the udder a substantial arteriovenous $^{14}CO_2$ difference was observed and it increased continuously with time to 6·37 counts/min./μmole. On the uninjected side of the udder the arteriovenous difference was much less. This was to be expected, since the specific activity of the [1-^{14}C]glucose was low on this side of the udder. There was very little ^{13}C in the CO_2, even in the blood from the injected side of the udder, and the excess of ^{13}C was too low to be detected in the remainder of the circulation. The ^{13}C content was low not because the [1-^{13}C]acetate was oxidized slowly; rather it was because of the large dilution of ^{13}C in the acetate and bicarbonate pools (see Table 4).

There was very little mixing of venous blood from the left and right sides of the udder, even though there are collateral connexions in the venous circulation of the udder (Turner, 1952). If there had been mixing of blood from the left and right sides of the udder, the blood from the two sides would have been more nearly alike and the specific activity of the CO_2 of the right subcutaneous abdominal vein would have been substantially above that of the posterior aorta.

The specific activity of the glucose of the blood from the subcutaneous abdominal vein increased with time from 2435 counts/min./μmole at 2·7 min. to 3075 at 4·3 min. There also was an increase in the percentage excess of ^{13}C in the acetate from 0·34 to 0·84 % during this time. The [1-^{14}C]glucose entering via the pudic artery has been estimated to have contained $5{\cdot}0 \times 10^3$ counts/min./μmole. Turner (1952) states that approximatey 7 l. of blood pass through the udder/min. in a cow yielding 20 l. of milk/day. The cow of this experiment produced about 22 l./day, and if 4 l. of blood passed through one-half of the udder and the blood contained 49 mg. of glucose/100 ml. (Baxter *et al.* 1955), there would have been 11×10^3 μmoles of glucose passing/min. Approximately 55×10^6 counts/min. were injected/min. as [1-^{14}C]glucose and therefore the calculated specific activity for glucose is

Table 3. *Arteriovenous difference in the specific activity of* CO_2 *of the blood during constant injection of* [1-^{14}C]*glucose and* [1-^{13}C]*acetate into the left pudic artery*

The experiment was done with the cow used in the experiment of Table 2 and there was 1·6 hr. between the two experiments. For this reason the blood contained about 1·57 counts/min./μmole of CO_2 at the start of the experiment. 12 ml. of a solution containing 150 μc of ^{14}C in 0·33 m-mole of [1-^{14}C]glucose and 3·2 m-moles of [1-^{13}C]acetate with 40 % excess of ^{13}C in the carboxyl group was injected at a constant rate into the left pudic artery during 4·5 min (approximately 2·7 ml./min.). During the injection blood was removed simultaneously from the posterior aorta, the jugular vein, the left subcutaneous abdominal vein and the right subcutaneous abdominal vein.

Time (min.)	1·2	2·7	4·3	2·7	4·3
	Counts/min./μmole of CO_2			Counts/min./μmole of glucose	
Posterior aorta	1·58	1·61	—	125	310
Jugular vein	1·58	1·57	1·63	—	—
Subcutaneous abdominal vein (injected side)	2·08	4·12	8·00	2435	3075
Subcutaneous abdominal vein (non-injected side)	1·65	1·66	1·86	—	—
Arteriovenous difference (injected side of udder)	0·50	2·51	6·37*	—	—
Arteriovenous difference (non-injected side of udder)	0·07	0·05	0·23*	—	—
		% Excess of ^{13}C in CO		% Excess of ^{13}C in C-1 of acetate	
Subcutaneous abdominal vein (injected side)	—	0·002	0·009	0·34	0·84

* Specific activity of the CO_2 of the blood from the jugular vein was used for these calculations.

$5{\cdot}0 \times 10^3$ counts/min./μmole. In the 4·3 min. about 17·2 ($4{\cdot}3 \times 4$) l. of blood would enter the injected side of the udder, and it seems likely that this quantity of blood would displace all the pre-injection blood from the injected half of the udder. The fall in specific activity of the blood glucose as it passed through the udder was apparently due to equilibration with unlabelled glucose of the tissue of the udder. The increase with time in the specific activity of the outflowing glucose would be expected as equilibration progressed. The increase cannot be accounted for by the return of ^{14}C from the general circulation, since there was little increase in the specific activity of the glucose of the posterior aorta (125 counts/min./μmole at 2·7 min. and 310 at 4·3 min.).

The estimated value for the carboxyl group of acetate was 3·8 % excess of ^{13}C. McClymont (1949, 1951) states there is about 10 mg. of acetate/100 ml. in the blood of cows. Therefore 7·42 m-moles of acetate were passing/min. Since 0·72 m-mole of acetate with 40 % excess of ^{13}C in C-1 was injected/min., the calculated excess of ^{13}C for the C-1 of acetate is 3·8 % ($0{\cdot}72/7{\cdot}42 \times 40{\cdot}0$). The highest value observed in the outflowing blood of the udder was 0·84 %. It would appear from this calculation that there was a very rapid decrease in the specific activity of the blood acetate which probably was caused by equilibration with unlabelled acetate present in the udder.

Unfortunately the data of Table 3 do not permit comparison of the rates of oxidation of C-1 of glucose and C-1 of acetate, because the excess of ^{13}C in the CO_2 was too low to permit accurate determination of the ^{13}C. For this reason an experiment was done with [1-^{14}C]acetate, in which the acetate was injected into the posterior aorta. This permitted direct measurement of the specific activity of the [1-^{14}C]acetate entering the udder via the pudic artery and thus avoided the necessity of calculating the ^{14}C activity on the basis of an assumed blood flow and acetate content in the blood. The procedure also avoided the possibility of dilution of the [^{14}C]acetate through collateral venous circulation, since both sides of the udder receive the labelled acetate from the posterior aorta.

The results are shown in Table 4 and confirm the observation that there is a decrease in the specific activity of the [1-^{14}C]acetate during the passage through the udder. The [^{14}C]acetate was diluted 2·5 (14 900/6080) times at 4 min. in the experiment of Table 4, whereas it was calculated to be diluted 4·5 (3·8/0·84) times at 4·3 min. in the experiment of Table 3.

Unfortunately only one set of values were obtained from the blood CO_2 (Table 4) and these were at 7 min. The specific activity of the CO_2 of the subcutaneous abdominal vein was 0·17 % [100 (15·4/8850)] of that of the [^{14}C]acetate of the vein. The value was 0·21 % [100 (6·37/3075)] (Table 3) with [1-^{14}C]glucose at 4·3 min. It would therefore appear that the C-1 of glucose and C-1 of acetate were oxidized to CO_2 at approximately the same rate. However, the comparison probably cannot be accepted at face value since the calculations involve a number of assumptions which may not be valid. For example, it is assumed the C-1 of glucose and C-1 of acetate undergo equal dilution of ^{14}C during their conversion into CO_2. It is probable, however, since the pools of intermediates of the Krebs cycle are interconvertible with amino acids and fatty acids, that the C-1 of acetate undergoes a greater dilution than does the C-1 of glucose in the intermediates of the pentose cycle. Further, the glucose and acetate may attain steady-state isotope concentrations at different rates with their counterparts in the tissues of the udder and this would lead to error. Clearly the comparison of the rates of oxidation of glucose and acetate oxidation is only a rough approximation.

Oxidation of [1-^{14}C]*glucose to* $^{14}CO_2$ *by blood* in vitro. As a control for the above experiments

Table 4. *Arteriovenous difference in the specific activity of the* CO_2 *and acetate of blood during the constant injection of* [1-^{14}C]*acetate into the posterior aorta*

A solution of [1-^{14}C]acetate (Volk) containing 25 μc of ^{14}C and 7·2 μmoles of acetate/ml. was injected at a rate of 3·2 ml./min. in the posterior aorta. Blood was collected from the left pudic artery and left subcutaneous abdominal vein. The CO_2 samples at 2 and 4 min. were lost.

		Time (min.)		
		2	4	7
Specific activity of acetate of blood (counts/min./μmole)	Pudic artery	10 400	14 900	10 100
	Subcutaneous abdominal vein	4 070	6 080	8 850
Arteriovenous difference of specific activity of acetate	—	6 330	8 820	1 250
Specific activity of CO_2 of blood (counts/min./μmole)	Pudic artery			16·5
	Subcutaneous abdominal vein			31·9
Arteriovenous difference of specific activity of CO_2	—			15·4

blood was incubated *in vitro* with [1-^{14}C]glucose. Quite unexpectedly it was found that the initial value of the $^{14}CO_2$ was high and that there then was a slow increase in the activity of $^{14}CO_2$. Further study showed that the initial evolution of $^{14}CO_2$ was due to a slight contamination of the [1-^{14}C]glucose with [^{14}C]-bicarbonate or -carbonate. The slow continuous evolution apparently was due to oxidation of [1-^{14}C]glucose by the blood cells. The results of these experiments are shown in Table 5. In experiment *A* at 1 min., the CO_2 contained 2·88 counts/min./μmole. There were 588 μmoles of CO_2 in the 20 ml. of blood; thus this is equivalent to 1693 counts/min. Each 20 ml. of blood contained 1 μc of [1-^{14}C]glucose, or $1{\cdot}65 \times 10^6$ counts/min. on the counting equipment used. Therefore, about 0·1 % of the counts added as glucose was CO_2.

In Table 1 values of 12·0 and 3·8 counts/min./μmole of CO_2 were obtained for the arteriovenous differences at 2 min. Since this is the time after termination of the injection of [1-^{14}C]glucose it is probable that most of the contaminating [^{14}C]-bicarbonate had passed out of the udder with the blood before collection of the blood sample. Therefore the results probably were not greatly affected by the presence of the ^{14}C contaminant in the [1-^{14}C]glucose. The slow oxidation of the [1-^{14}C]-glucose by the blood *in vitro* gave a CO_2 value of 12·6 counts/min./μmole at 40 min. in Expt. *A*, Table 5. A comparable value would not be expected for the experiment *in vivo* because the conditions are quite different. In Expt. *A*, Table 5, there were 10 μc of [1-^{14}C]glucose in 200 ml. of blood which contained approximately 100 mg. of glucose. In the experiment *in vivo* 100 μc of [1-^{14}C]glucose was injected and passed through the udder and was distributed in the body glucose pool. Baxter *et al.* (1955) have calculated that the body pool in a 457 kg. cow contains 62·4 g. of glucose. Thus after the [1-^{14}C]glucose entered the general circulation it was diluted many times more than in the experiment *in vitro*.

Expt. *B* was designed to serve as a control for the experiment of Table 3. The glucose contained 5000 counts/min./μmole, which was comparable with that of the glucose which flowed through the left side of the udder in the experiment of Table 3. It is seen that the CO_2 at zero time contained 0·22 count/min./μmole, and that there was very little increase in the specific activity of the CO_2 during 4·5 min. of incubation. There was some increase in 15 min. Apparently a correction value of less than 0·22 count/min./μmole should be subtracted from the specific activity of CO_2 from the left subcutaneous abdominal vein of Table 3. It would be less than 0·22 because equilibration with unlabelled bicarbonate pool would lower the specific activity of the CO_2 (Table 2). This correction would not alter significantly the value of 6·37, which was obtained for the arteriovenous difference at 4·3 min. It therefore is concluded that the [1-^{14}C]glucose was oxidized to $^{14}CO_2$ by the udder and that the oxidation by blood and the contamination by $^{14}CO_2$ contributed very little to the activity of ^{14}C observed in the CO_2.

SUMMARY

1. The metabolism of the lactating cow's udder was investigated by determinations of arteriovenous differences in the isotope concentrations of $^{14}CO_2$ or $^{13}CO_2$ of the udder during a continuous injection of [1-^{14}C]acetate or [1-^{14}C]glucose and [1-^{13}C]acetate. The isotope concentrations of the blood glucose and acetate were also determined in the venous blood of the udder.

2. The relative rates of oxidation of the C-1 of glucose and of the C-1 of acetate to CO_2 were compared on the basis of the ratios of the isotope concentrations of the substrates and of the CO_2. By

Table 5. *Oxidation of* [1-^{14}C]*glucose to carbon dioxide by blood* in vitro

Experiment A. 250 ml. of blood was drawn from the jugular vein of a cow into 500 units of heparin. 10 μc of [1-^{14}C]-glucose (9·2 mg.) in 10 ml. of aq. 0·09 % NaCl was added to 200 ml. of the blood and the mixture was incubated at 37° in a closed flask. At the indicated times 20 ml. of blood was withdrawn and the CO_2 was liberated and collected.

Experiment B. 100 ml. of blood was drawn into a syringe containing a marble for mixing of the blood and glucose. 5 ml. of a solution of [1-^{14}C]glucose containing 0·78 mg. of glucose and 0·85 μc of ^{14}C was drawn into the syringe. The mixed solution was incubated at 37° in the syringe and at the indicated times CO_2 was liberated from 20 ml. of blood. The zero-time CO_2 was obtained by adding 1 ml. of the [1-^{14}C]glucose to acid solution, and then citrate and 19 ml. of blood were added to the acid solution.

Expt. *A*				Expt. *B*	
Time (min.)	CO_2 (counts/min./μmole)	Time (min.)	CO_2 (counts/min./μmole)	Time (min.)	CO_2 (counts/min./μmole)
1	2·88	5	3·34	0	0·22
2	2·85	10	4·18	1·8	0·23
3	2·90	20	6·97	4·5	0·25
4	3·14	40	12·60	15	0·43

this method C-1 of acetate was estimated to be oxidized to CO_2 in the udder at about the same rate as is C-1 of glucose. This estimate may be subject to considerable error because of assumptions made in the calculation.

3. [1-^{14}C]- and [6-^{14}C]-Glucose were alternately injected into the pudic artery of a cow and the specific activities of the CO_2 of the venous blood of the udder were determined. After injection of [1-^{14}C]glucose the specific activity of the CO_2 was much greater than it was after [6-^{14}C]glucose. These results indicate that the pentose cycle occurs in the udder of the lactating cow.

4. The equilibration between the glucose, acetate and bicarbonate of the blood and that of the pools of the udder was investigated. This equilibration alters the specific activity of the compounds and makes it difficult to apply tracer-dilution methods in the intact animal.

A large group of students and staff from the Department of Dairy Science and from the College of Veterinary Medicine assisted in collecting the blood samples and in liberating the CO_2. Their assistance is gratefully acknowledged. The ^{13}C determinations were done at the University of Minnesota and the authors thank Dr Nathan O. Lifson for assistance in these measurements.

This investigation has been supported by grants from the Atomic Energy Commission under Contract No. AT-(30-1)-1320 at Western Reserve University and Contract No. AT-(11-1)-67 at the University of Illinois. The ^{14}C was obtained on allocation from the U.S. Atomic Energy Commission.

REFERENCES

Abraham, S., Cady, P. & Chaikoff, I. L. (1957). *J. biol. Chem.* **224**, 955.
Abraham, S., Hirsch, P. F. & Chaikoff, I. L. (1954). *J. biol. Chem.* **211**, 31.
Baker, N., Shreeve, W. W., Shipley, R. A., Incefy, G. E. & Miller, M. (1954). *J. biol. Chem.* **211**, 575.
Barcroft, J., McAnally, R. A. & Phillipson, A. T. (1944). *J. exp. Biol.* **20**, 120.
Baxter, C. F., Kleiber, M. & Black, A. L. (1955). *Biochim. biophys. Acta*, **17**, 354.
Bernstein, W. & Ballentine, R. (1950). *Rev. sci. Instrum.* **21**, 158.
Black, A. L., Kleiber, M., Butterworth, E. M., Brubacher, G. B. & Kaneko, J. I. (1957). *J. biol. Chem.* **227**, 537.
Duncombe, W. G. & Glascock, R. F. (1956). *Biochem. J.* **63**, 326, 332.
Espe, D. (1948). In *Secretion of Milk*, p. 24. Ames: Iowa State College Press.
Glock, G. E. & McLean, P. (1953). *Biochim. biophys. Acta*, **12**, 590.
Glock, G. E. & McLean, P. (1954). *Biochem. J.* **56**, 171.
Glock, G. E., McLean, P. & Whitehead, J. K. (1956). *Biochim. biophys. Acta*, **19**, 546.
Kleiber, M., Black, A. L., Brown, M. A., Baxter, C. F., Luick, J. R. & Stadtman, F. H. (1955). *Biochim. biophys. Acta*, **17**, 252.
Lachance, J. P., Popják, G. & de Waard, A. (1958). *Biochem. J.* **68**, 7P.
McClymont, G. L. (1949). *Biochem. J.* **45**, i.
McClymont, G. L. (1951). *Aust. J. agric. Res.* **2**, 158.
Nelson, N. (1944). *J. biol. Chem.* **153**, 375.
Nier, A. O. (1947). *Rev. sci. Instrum.* **18**, 398.
Peeters, G. & Debackere, M. (1956). *Arch. int. Physiol. Biochim.* **64**, 527.
St. Clair, L. E. & Hardenbrook, H. J. (1956). *J. Amer. vet. med. Ass.* **129**, 405.
Schambye, P., Wood, H. G. & Kleiber, M. (1957). *J. biol. Chem.* **226**, 1011.
Seubert, W., Greult, G. & Lynen, F. (1957). *Angew. Chem.* **69**, 359.
Somogyi, J. (1945). *J. biol. Chem.* **160**, 61.
Swim, H. E. & Krampitz, L. O. (1954). *J. Bact.* **67**, 426.
Turner, C. W. (1952). In *The Mammary Gland*, p. 56. Columbia: Lucas Bros.
Van Slyke, D. D. & Folley, S. J. (1940). *J. biol. Chem.* **136**, 509.
Wood, H. G., Gillespie, R., Joffe, S., Hansen, R. G. & Hardenbrook, H. (1958*a*). *J. biol. Chem.* **233**, 1271.
Wood, H. G., Joffe, S., Gillespie, R., Hansen, R. G. & Hardenbrook, H. (1958*b*). *J. biol. Chem.* **233**, 1264.
Wood, H. G., Siu, P. & Schambye, P. (1957). *Arch. Biochem. Biophys.* **69**, 390.

Phospholipid Metabolism in Nervous Tissue

1. A RECONSIDERATION OF BRAIN AND PERIPHERAL-NERVE PHOSPHOLIPID METABOLISM *IN VIVO*

By A. N. DAVISON and J. DOBBING
Department of Pathology, Guy's Hospital Medical School, London, S.E. 1

(*Received* 20 *April* 1959)

Experiments designed to investigate the metabolic activity of the chemical components of myelin have led to a suggestion that these substances, once incorporated into the nerve sheath during development, may remain there unchanged for long periods in relation to the lifetime of the animal. Thus [4-^{14}C]cholesterol injected into the residual yolk sac of the developing chick (Davison, Dobbing, Morgan & Wright, 1958) or intraperitoneally into the developing rabbit (Davison, Dobbing, Morgan & Wright, 1959) is slowly and continuously incorporated into the brain during myelination, much of it remaining there for a year or more with little or no change in quantity or molecular configuration

(Davison & Wajda, 1959). A proportion of the cholesterol taken up into the brain in the early stages does, however, exhibit turnover, but since this occurs predominantly in the grey matter, it has been regarded as associated with cellular metabolic processes rather than with myelination. Similarly, when [3-^{14}C]serine is administered to young rabbits (Davison, Morgan, Wajda & Wright, 1959) a small proportion becomes incorporated into brain lipids, considered to be predominantly in myelin, and remains there virtually unchanged well into adult life.

The apparent stability of some of the brain phospholipids demonstrated in the last-named experiment, together with earlier results (Changus, Chaikoff & Ruben, 1938) for total brain phospholipids, seemed to conflict with the now widely held view (Dawson & Richter, 1950; Dawson, 1953, 1954; Streicher & Gerard, 1954; McAlpine, Compston & Lumsden, 1955; Richter, 1955; Ansell & Dohmen, 1957; McIlwain, 1959) that these undergo rapid turnover at a rate, according to some of these authors, equivalent to complete replacement in 70 hr. These authors were, of course, fully aware that an average turnover rate means no more than that some part of the material is turning over, and gives no information about the range of rates to be found in the mixture; and that therefore a rapid average rate is entirely consistent with great (possibly infinite) stability of some of the material. However, the impression seems to be widespread that phospholipid turnover in brain is comparable with that in other tissues. It was decided to reinvestigate these aspects of brain-phospholipid metabolism with ^{32}P *in vivo*, and to determine the degree of persistence of these substances after their administration to neonatal animals for much longer periods of time than previous workers have employed. Since the metabolism of the cholesterol of the peripheral nervous system appears to be similar to that in the brain (Davison, Dobbing, Morgan & Wright, 1959), the present study of brain phospholipids has been extended to the sciatic nerve and, for comparison and contrast, to other non-nervous tissues. Preliminary reports of some aspects of this work have already appeared (Davison & Dobbing, 1958, 1959).

METHODS

Animals. Four litters of 16-day-old white Wistar rats, with an average weight of 30–35 g., were given an intraperitoneal injection of 25 μC of ^{32}P as phosphate in 0·25 ml. of 0·9% NaCl.

Two methods for the preparation of phospholipid extracts have been used. In the first technique (Method 1), lipids are extracted in $CHCl_3$–methanol (2:1, v/v) as recommended by Folch, Ascoli, Lees, Meath & LeBaron (1951). In the second technique (Method 2), acid-soluble constituents are first removed before the precipitated phospholipids are extracted with organic solvents (Ansell & Dohmen, 1956).

Method 1: *extraction of lipid.* Rats were killed at intervals by intraperitoneal injection of pentobarbitone. The brain was removed immediately and, after weighing, homogenized in 20 vol. of $CHCl_3$–methanol (2:1, v/v). The filtered extract was used for the measurement of radioactivity of total phospholipid. Both sciatic nerves were dissected out and freed from adherent fat and tissues. The nerve was minced with scissors and homogenized in 20 vol. of $CHCl_3$–methanol (2:1, v/v). Other tissues were removed and treated in a similar way.

In some experiments the crude lipid extract was further washed with 0·2 vol. of water according to the method of Folch, Lees & Sloane-Stanley (1957). Radioactivity was measured in this washed extract and also in the upper phase obtained on separation. Proteolipid A and B were decomposed by evaporating the wet $CHCl_3$–methanol extract to dryness three times; the lipid was dissolved in $CHCl_3$–methanol and filtered (Folch & Lees, 1951).

Method 2: *extraction of acid-soluble material and phospholipid.* Immediately after death the brain was dispersed in 20 ml. of ice-cold 5% (w/v) trichloroacetic acid solution and allowed to stand for 30 min. at 0°. A clear extract was obtained subsequently by centrifuging at 0° for 15 min. at 25 000 *g*. The precipitate was resuspended in 20 ml. of 5% (w/v) trichloroacetic acid solution and reprecipitated by centrifuging. This procedure was continued until radioactivity was virtually absent in the supernatant. The residual phospholipid precipitate was macerated in 5 ml. of acetone; $CHCl_3$–methanol (2:1, v/v) was then added, the whole was homogenized, filtered and the volume adjusted with $CHCl_3$–methanol (2:1, v/v) to 20 times the original brain weight (20 ml./g. of brain).

Acid-soluble material in blood. The method of Dawson & Richter (1950) was used.

Phosphorus analyses. These were performed on samples oxidized with 1 ml. of 60% perchloric acid until clear, as described by Strickland, Thompson & Webster (1956).

Radioactivity determination. Counts were made on 10 ml. of a suitable dilution of extract in a type M6 β-counter for liquids. Necessary corrections for background and decay were applied.

RESULTS

When lipids were extracted in chloroform–methanol, it was found that on washing the extract with water only traces of phosphorus (see also Uzman & Rumley, 1958) and less than 2·5% of the radioactivity were present in the separated upper phase 14 days after injection. Similarly decomposition of proteolipid (Folch & Lees, 1951) had little effect on the level of radioactivity of chloroform–methanol extract. For these reasons phospholipid radioactivity and phosphorus levels subsequent to the fourteenth day after injection, recorded in this paper, are those of the crude unwashed chloroform–methanol extract.

Series 1. The body weights, with brain and other organ weights, of the first series of rats are shown in Table 1. All survived the rather large dose of ^{32}P and gained weight at a normal rate.

To avoid complicated corrections for the dilution effect of growth and possible changes in water content in the brain during myelination (Uzman & Rumley, 1958) it is convenient to express brain radioactivity in terms of the whole organ. Whole-organ phospholipid radioactivity in brain and liver in the first series of rats is shown in Fig. 1. Maximum phospholipid radioactivity in liver is achieved during the day of injection and values thereafter show a very rapid decline. In the brain, however, incorporation of ^{32}P is slower, maximum values are reached only after about 2 weeks, and subsequent decline is extremely slow. At the end of the experiment, 70 days after injection, there is no residual radioactivity in liver, kidney or blood of the rats (which now weighed about 180 g.), whereas in the brain a considerable proportion of the incorporated ^{32}P remains.

The results for sciatic nerve in Fig. 2 are expressed in terms of radioactivity per gram wet weight in view of the difficulty of defining the anatomical structure, and appear to be in general agreement with the results for the central nervous system. This method of expressing results as concentrations unfortunately tends to mask the degree of persistence in growing animals because of the dilution effect of growth. The kidney results in Fig. 2 are given here for comparison and are therefore also expressed as radioactivity/g. wet wt.

Series 2. The second experiment was designed to re-investigate the fate of ^{32}P immediately after the injection, and particularly its distribution in blood and brain acid-soluble phosphorus and brain phospholipid. The results in Fig. 3 show the expected early appearance of acid-soluble ^{32}P in the blood, followed by a rapid fall in its radioactivity. The rate of increase of acid-soluble ^{32}P in whole brain is slower than the rate of increase of blood acid-soluble ^{32}P in the first 4 hr. after injection. From this time until the end of the first day, the brain acid-soluble ^{32}P continues to rise, in spite of a precipitous fall in blood acid-soluble phosphorus radioactivity during this time. The rate of increase of whole-brain phospholipid ^{32}P is considerably slower in the first 7 hr. than the rate of increase of

Table 1. *Whole body and organ weights of first series of rats injected with* ^{32}P *when* 16 *days old*

Age (days)	Weight (g.) Whole body	Brain	Liver	Kidneys
17	35	1·04	2·12	0·48
21	58	1·41	3·42	0·75
23	50	1·27	2·93	0·60
27	61	1·48	3·4	0·80
30	75	1·31	3·84	0·58
37	109	1·48	6·46	1·14
44	130	2·6	7·1	1·32
51	120	1·55	6·94	1·03
58	184	1·64	11·37	1·71
65	185	1·70	10·82	1·84
72	180	1·70	8·94	1·55
79	175	1·74	10·59	1·49
86*	180*	1·79*	10·11*	1·68*

* Mean of values from four rats.

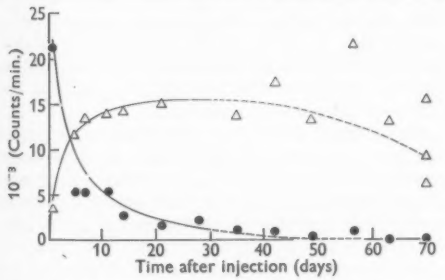


Fig. 1. Radioactivity in phospholipids of whole brain (△) and whole liver (●) in the first series. Rats were injected with [^{32}P]phosphate when 16 days old. Results for liver have been divided by four.

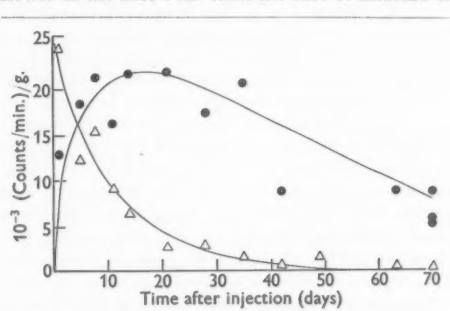


Fig. 2. Radioactivity in phospholipids of sciatic nerve (●) and kidney (△) of the first series. Results are expressed as counts/min./g. and have been multiplied by two for the kidney.

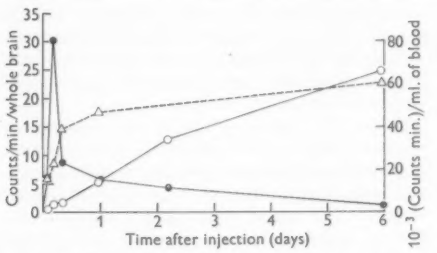


Fig. 3. Radioactivity of blood (●) and brain (△) acid-soluble extract and radioactivity of brain phospholipid (○) after injection of ^{32}P in the second series of rats when 16 days old.

Table 2. *Brain weights, acid-soluble and phospholipid phosphorus and 'relative specific activities' of second series of rats injected with* ^{32}P *when* 16 *days old*

Time after injection (hr.)	Brain wt. (g.)	Brain phosphorus (mg./whole organ)		Relative specific activity [counts/min./mg. of brain phospholipid P / counts/min./mg. of brain acid-soluble P]
		Acid-soluble P	Phospholipid P	
2	1·12	0·84	2·40	0·044
4	1·20	0·80	2·57	0·058
7	1·16	0·76	1·30	0·086
144	1·37	0·85	1·96	0·470

whole-brain acid-soluble ^{32}P, but, like the latter, it continues to rise in spite of falling blood values. Towards the end of the 6-day period studied, since the rate of increase of brain acid-soluble ^{32}P has declined more than the rate of increase of brain phospholipid ^{32}P the values of the latter eventually exceed those of the former.

The brain weights of the second series, the phospholipid and acid-soluble phosphorus content of the brain extracts and acid-soluble phosphorus content of the blood, together with the 'relative specific activities' calculated according to Dawson & Richter (1950), are shown in Table 2.

DISCUSSION

The work presented here is a preliminary investigation of the apparent discrepancy between our recent results (Davison, Morgan, Wajda & Wright, 1959), which suggest a remarkable degree of permanence of those brain phospholipids which are mainly present in the myelin sheath, and the now widely held view that the brain phospholipids as a whole undergo replacement at a rate more comparable with the rates in other tissues. The present preliminary experiments differ from those of many of the earlier workers, first, in that the labelled phosphorus has been incorporated into the brain phospholipids *in vivo* during a period of growth when development of the nervous system, and particularly of the myelin sheaths, is known to be taking place most rapidly, and, secondly, in that the animals have been allowed to survive until they have reached adult weight, when blood and other tissue levels of injected isotope have declined to zero and the central nervous system may virtually be regarded as fully developed.

The most striking feature of the results is the apparent difference of behaviour shown between the phospholipids of the brain and peripheral nerve on the one hand, and the blood and other tissues on the other. The well-known delay in the passage of ^{32}P from blood to brain, compared with its rapid movement into other tissues, is followed by a marked retention of phosphorus incorporated into brain and peripheral-nerve phospholipids, compared with the rapid loss of label from the liver and kidney.

It is beyond the scope of this discussion to analyse the nature of the so-called 'blood–brain barrier'. It has been pointed out elsewhere (Dobbing, 1956) that attempts to define such a barrier in terms of a single anatomical structure for all substances have failed, and that for those substances concerned in normal metabolic processes the 'barrier' phenomenon must increasingly be regarded as a reflexion of, rather than a limiting factor in, brain metabolism *in vivo*. As can be seen from the relative rates of increase of acid-soluble ^{32}P in blood and brain during the first 4–7 hr. (Fig. 3), there is a delayed entry of ^{32}P from the blood into the brain. During the same first few hours, there is a very much greater delay in incorporating the brain acid-soluble ^{32}P into phospholipid. If it can be assumed that the blood acid-soluble ^{32}P passes into the brain in acid-soluble form and is subsequently incorporated within the brain into phospholipids, then it would appear that the principal delay in the process of incorporation of blood acid-soluble ^{32}P into brain phospholipids occurs within the brain itself. In other words, the slow rate of appearance of the label in the brain phospholipids seems to be limited by the rates of their synthesis by the nervous tissue, and not so much by the rate of entry of precursor ^{32}P into the brain. The delay of incorporation of blood acid-soluble ^{32}P into brain phospholipid cannot therefore be wholly attributed to the interposition of a blood–brain barrier. Moreover, the apparent similarity in the pattern of uptake into and retention of phosphorus by sciatic nerve (Fig. 2) would imply a structural blood–tissue barrier for the peripheral nervous system such as has been postulated in the past for the brain, and this seems unlikely.

The classical concept of the blood–brain barrier has led to the widespread adoption of 'relative specific activity' (specific activity of brain-phospholipid phosphorus/specific activity of brain acid-soluble phosphorus) as a means of investigating rates of phospholipid synthesis in the brain *in vivo* (Dawson & Richter, 1950). By expressing the specific activity of the brain-phospholipid phosphorus in terms of the specific activity of brain acid-soluble phosphorus at the same point in time, it has been sought to annul the effects of the 'barrier',

and the changing values of this relative specific activity after single doses of ^{32}P have in this way been taken as estimates of rates of phospholipid synthesis (Ansell & Dohmen, 1957). The relative specific activities at single points in time, which together form the curve from which synthetic rates are deduced, are, however, open to some objection under certain circumstances. As can be seen from Fig. 3, the whole-brain phospholipid ^{32}P level at any given time during the first day is not due to the precursor acid-soluble phosphorus level at that same point in time. Because of the delay in incorporation of precursor into product, the amount of product at any time is in fact related to a precursor level at some time previously. The greater the rate of change of product and precursor levels, the greater will be the error in the implications of the relative specific activity expression because under these circumstances the delay in the synthetic process is more apparent. It is for this reason that the error is greatest in the earliest stages of a single-dose experiment, because at this time the rate of change of precursor and product levels is maximal. The result of the error in the relative specific activity as usually expressed would therefore be to underestimate the apparent rate of synthesis of the brain phospholipid as a whole; and if this higher, corrected rate of synthesis were extrapolated to an expression of even shorter turnover time, this would be still more difficult to reconcile with the degree of metabolic stability which we have demonstrated here.

If relative specific activities are calculated in the first few hours of the present experiments, values reached are in reasonable agreement with those reported by other workers (Dawson & Richter, 1950; Ansell & Dohmen, 1957); this is in spite of our results having been obtained in neonatal, rapidly developing animals whose phospholipid metabolism might be expected to be quite different from that of the adult mice used by Dawson & Richter.

In further consideration of this complex problem, account must be taken of the great differences in rates of synthesis between the various components of the brain-phospholipid mixture represented by the chloroform–methanol extract (Ansell & Dohmen, 1957). Although differences exist between some findings *in vivo* and *in vitro* in this respect (Dawson, 1954; Streicher & Gerard, 1954; Strickland, 1952), it seems likely that these are due to the difficulties in reproducing *in vivo* conditions. The observation *in vivo* of Ansell & Dohmen (1957) that, in the brain, diphosphoinositide is synthesized considerably more quickly than is phosphatidylserine (other phospholipids being formed at intermediate rates) is well established. The fact that these varying rates of synthesis are expressed as relative specific activities does not affect the relationships, since the denominator at each point in time is the same for all members of the group. It could be postulated that the relative stability of brain phospholipids apparent in our results might simply represent those lipids whose rates of synthesis are known to be slow, and whose similarly slow rates of turnover would be expected to result in some degree of apparent persistence. It is indeed likely that much of the apparent persistence may be explained in this way, since the slowly metabolized components of the mixture are probably largely identical with those components concerned with myelin-sheath structure. However, a rough calculation, making many assumptions, suggests that the degree of persistence demonstrated here is much more than could be accounted for by the slow rate of synthesis of phosphatidylserine.

The degree of persistence already shown in brain and sciatic nerve in the presence of negligible levels of labelled phosphorus in liver and kidney, taken in conjunction with the finding of persistence of certain ^{14}C-labelled 'myelin' phospholipids and cholesterol in previous work, leads to the general hypothesis that these substances, once incorporated into the myelin sheath in the course of development, may persist there with little or no alteration as long as the sheath itself survives. If this be true, it will never be possible to assess total turnover times by extrapolation of short-term measurements of rates of synthesis, however valid the assumptions may be with regard to the precursor–product relationship; furthermore, no experiments can continue long enough *in vitro* to demonstrate this effect, except, perhaps, in tissue culture.

The provisional hypothesis is that brain constituents, in common with the constituents of other tissues, may be roughly divided into at least two main groups according to their predominant rates of turnover. The total 'chloroform–methanol extract' value at any time during the earlier stages of the experiment is the sum of the structural, more permanent lipids and the non-structural lipids, which are more labile. It is unlikely that a complete separation can be made by either biochemical or anatomical techniques, since it is probable that individual components are each distributed between both compartments in varying degree.

SUMMARY

1. The fate of injected ^{32}P has been followed in the phospholipids of the brain, sciatic nerve and other tissues of developing rats for a period of 70 days.

2. The degree of stability of the phospholipids as a whole in both central and peripheral nervous tissue contrasts markedly with their more rapid turnover in other tissues.

3. The relative stability demonstrated *in vivo* is discussed in the light of currently conflicting views of phospholipid turnover in brain.

4. Some aspects of the blood–brain barrier for phosphorus have been reconsidered.

5. It is suggested that the stability of these phospholipids, together with the stability of other brain lipids previously reported, may reflect their structural role in the myelin sheath.

The authors wish to thank Miss Jennifer Vogt for her valuable technical assistance.

REFERENCES

Ansell, G. B. & Dohmen, H. (1956). *J. Neurochem.* **1**, 150.
Ansell, G. B. & Dohmen, H. (1957). *J. Neurochem.* **2**, 1.
Changus, G. W., Chaikoff, I. L. & Ruben, S. (1938). *J. biol. Chem.* **126**, 493.
Davison, A. N. & Dobbing, J. (1958). *Lancet*, ii, 1158.
Davison, A. N. & Dobbing, J. (1959). *Biochem. J.* **7**, 10P.
Davison, A. N., Dobbing, J., Morgan, R. S. & Wright, G. P. (1958). *J. Neurochem.* **3**, 89.
Davison, A. N., Dobbing, J., Morgan, R. S. & Wright, G. P. (1959). *Lancet*, i, 658.
Davison, A. N., Morgan, R. S., Wajda, M. & Wright, G. P, (1959). *J. Neurochem.* **4**, 353.
Davison, A. N. & Wajda, M. (1959). *Nature, Lond.*, **183**, 1606.
Dawson, R. M. C. (1953). *Biochem. J.* **55**, 507.
Dawson, R. M. C. (1954). *Biochem. J.* **57**, 237.
Dawson, R. M. C. & Richter, D. (1950). *Proc. Roy. Soc.* B, **137**, 252.
Dobbing, J. (1956). *Guy's Hosp. Rep.* **105**, 27.
Folch, J., Ascoli, I., Lees, M., Meath, J. A. & LeBaron, F. N. (1951). *J. biol. Chem.* **191**, 833.
Folch, J. & Lees, M. (1951). *J. biol. Chem.* **191**, 807.
Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957). *J. biol. Chem.* **226**, 497.
McAlpine, D., Compton, M. D. & Lumsden, C. E. (1955). *Multiple Sclerosis.* London: E. and S. Livingstone Ltd.
McIlwain, H. (1959). *Biochemistry and the Central Nervous System*, 2nd ed. London: J. and H. Churchill Ltd.
Richter, D. (1955). *Biochemistry of the Developing Nervous System*, p. 225. Ed. by Waelsch, H. New York: Academic Press Inc.
Streicher, E. & Gerard, R. W. (1954). *Proc. Soc. exp. Biol., N.Y.*, **85**, 174.
Strickland, K. P. (1952). *Canad. J. med. Sci.* **30**, 484.
Strickland, K. P., Thompson, R. H. S. & Webster, G. R. (1956). *J. Neurol. Psychiat.* **19**, 12.
Uzman, L. L. & Rumley, M. K. (1958). *J. Neurochem.* **3**, 171.

The Synthesis of Nicotinuric Acid by Rat-Kidney Preparations

By K. M. JONES and W. H. ELLIOTT
Department of Biochemistry, University of Oxford

(*Received* 14 *April* 1959)

Nicotinuric acid (nicotinoylglycine) was first isolated by Ackermann (1912) from the urine of a dog which had been fed with nicotinic acid, and it has since been found in the urine of other species, including the rat (Huff & Perlzweig, 1942) and man (Melnick, Robinson & Field, 1940). More recent work has shown that it is present only in small quantities in rat urine either normally or after the administration of nicotinamide, but that it is an important urinary metabolite when doses of nicotinic acid are given, especially at short time intervals after administration (Reddi & Kodicek, 1953; Leifer, Roth, Hogness & Corson, 1951; Johnson & Lin, 1953; Lin & Johnson, 1953). No study of nicotinuric acid synthesis *in vitro* has been reported.

In the course of exploratory experiments on nicotinic acid metabolism *in vitro* it was observed that a substance corresponding chromatographically with nicotinuric acid was formed by various tissue preparations. In this paper the identification of the compound with nicotinuric acid and its synthesis by rat-kidney preparations are described. A preliminary report of part of the work has been published previously (Jones & Elliott, 1954).

EXPERIMENTAL

Materials

Nicotinuric acid. This was synthesized by the method of Rohrlich (1951), with methyl nicotinate prepared as described by Levine & Snead (1951).

Nicotinic acid. Commercial nicotinic acid was purified by boiling with decolorizing charcoal and crystallizing it twice from water.

Adenosine triphosphate. The dibarium salt of adenosine triphosphate (ATP) was prepared by the method of Needham (1942) and converted into monobarium ATP (Kerr, 1941). For use, solutions of potassium ATP were prepared by removing the barium with Amberlite IR-100 (H form) and neutralizing to pH 6·8 with KOH. In later experiments crystalline disodium ATP (Schwarz Laboratories Inc., Mount Vernon, N.Y., U.S.A.) was used. It was dissolved in water and neutralized to pH 6·8 with KOH. Solutions of ATP contained added KCl to make them isotonic with rat serum.

Phosphate buffers. These were prepared from solutions of KH_2PO_4 and KOH.

Methods

Chromatography of tertiary nicotinic acid derivatives. Nicotinic acid derivatives having a tertiary ring nitrogen (i.e. nicotinic acid, nicotinamide and nicotinuric acid) were separated by paper chromatography with butan-1-ol saturated with water (butan-1-ol–water) as solvent (Kodicek & Reddi, 1951). The compounds were detected by two methods: procedure (1), by their u.v. absorption, either by direct observation or by contact photography (Markham & Smith, 1949) with unfiltered light from a mercury lamp; procedure (2), by the König reaction (Kodicek & Reddi, 1951), for which the dried paper was placed in an atmosphere of cyanogen bromide for 1 hr. and then sprayed with a 2% solution of *p*-aminobenzoic acid in 0·75 N-HCl–ethanol (3:1, v/v).

Measurement of nicotinuric acid synthesis. The standard incubation mixture used in all experiments, except those with tissue slices, had the following composition: 0·3 ml. of 0·1 M-phosphate buffer, pH 7·35; 0·03 ml. of 0·1 M-$MgCl_2$; 0·6 ml. of 0·1 M-potassium fumarate; 0·15 ml. of 0·01 M-ATP; 0·1 ml. of 0·154 M-potassium nicotinate; 0·2 ml. of 0·308 M-glycine; 1 ml. of the tissue preparation; 0·154 M-KCl to a total volume of 3·08 ml. Incubations were performed in conical flasks or Warburg manometers shaken at 38° for 2 hr. with a gas phase of air.

After incubation, the mixtures were placed in a boiling-water bath for 5 min., cooled and centrifuged. Samples (0·308 ml.) of the supernatants were taken for the estimation of nicotinuric acid by the method of Jones (1959*b*). Alternatively, after chromatography of the samples, nicotinuric acid was detected qualitatively by procedure (2) above.

Nicotinamide. This was estimated by the method of Jones (1959*b*).

Total nitrogen. This was estimated by the micro-Kjeldahl method.

Preparation of homogenates. Kidneys from female albino rats were washed and cooled in ice-cold 0·154 M-KCl or 0·25 M-sucrose. After cooling, the medulla was cut away and the kidney cortex blotted, weighed and homogenized in 9 vol. of ice-cold fluid in a Potter & Elvehjem (1936) homogenizer. The homogenate was filtered through either several layers of muslin or a single layer of flannellette before use.

Preparation of tissue fractions. The following procedure, modified from that of Schneider (1948), was used to fractionate homogenates into nuclear, mitochondrial, microsome and soluble fractions. All operations were performed at 0–2°.

Kidney-cortex homogenate was prepared with 8·5 vol. of 0·25 M-sucrose plus 0·5 vol. of 0·1 M-potassium ethylenediaminetetra-acetate (EDTA), pH 7·4. A sample of the filtered homogenate was set aside at 0° for experiments on the whole homogenate. The remainder of the filtered homogenate was centrifuged at 800 *g* (average value) for 10 min. at 0°. The supernatant was decanted and kept in the cold. The sediment was resuspended in a volume of 0·25 M-sucrose equal to half that of the homogenate used and again centrifuged at 800 *g* for 10 min. at 0°. The supernatant was decanted and the sediment suspended in a suitable volume of 0·25 M-sucrose (nuclear fraction). The combined supernatants from these centrifugings were centrifuged at 5000 *g* (average value) for 10 min. at 0°. The supernatant was decanted and kept in the cold. The sediment was resuspended in a volume of 0·25 M-sucrose equal to half that of the homogenate originally taken and again centrifuged at 5000 *g* for 10 min. at 0°. The supernatant was decanted and the sediment taken up in a suitable volume of 0·25 M-sucrose (mitochondrial fraction). The combined supernatants from the sedimentation and washing of the mitochondria were then centrifuged at 100 000 *g* (average value) for 30 min. at 0°. The sediment was taken up in 0·25 M-sucrose (microsome fraction). The supernatant from this centrifuging was used as the soluble fraction. In some experiments the microsome and soluble fractions were not tested separately, and in this event the combined supernatants from the sedimentation of the mitochondrial fraction were used as the microsome plus soluble fraction.

Preparation of mitochondrial suspensions. The mitochondrial fraction was sedimented and washed as described above and the final pellet suspended in a volume of 0·154 M-KCl equal to one-fifth of that of the homogenate used. This gave suspensions containing about 1 mg. of total N/ml.

RESULTS

Identification of the nicotinic acid metabolite

The substance formed from nicotinic acid by incubation with slices or homogenates of rat kidney or liver had the same R_F in butan-1-ol–water and in acetone–water (85:15, v/v), and gave the same colours on treatment by procedure (2) as a sample of synthetic nicotinuric acid.

For more rigorous identification the compound was isolated by paper chromatography. Samples of incubation mixtures were applied in a band to Whatman no. 3 paper and the chromatograms developed for 40–48 hr. with butan-1-ol–water. The compound was located by its u.v. absorption, the band was cut out and eluted chromatographically with water. Narrow strips of the chromatograms were subjected to procedure (2) or treated with ninhydrin to show that the compound had been separated from nicotinic acid and from ninhydrin-positive compounds. The eluate was again chromatographed in a band for 24 hr. and the compound eluted as before. This second eluate was used in the subsequent identification.

The spectra of samples of the compound and of synthetic nicotinuric acid were compared over the range 230–280 mμ after chromatography in butan-1-ol–water for 12 hr. and elution from the paper. Separate samples were eluted with N-HCl and with water. The spectrum of the biologically-formed compound was identical in both solvents with that of synthetic nicotinuric acid (Fig. 1).

Further samples of the biosynthetic compound were hydrolysed in 6 N-HCl at 105° for 18 hr. in sealed tubes and spotted on to paper as described by Consden, Gordon & Martin (1947). Chromatograms developed with butan-1-ol–water and with acetone–water (85:15, v/v) showed the presence in the hydrolysate of nicotinic acid. Another chromatogram was developed in two dimensions with

phenol–0·3 % NH_3 followed by methanol–pyridine–water (80:2:20, by vol.), and sprayed with ninhydrin. A single spot having the same R_F as glycine was found. A duplicate chromatogram on which glycine was added to the hydrolysate also showed a single spot, reinforced in intensity. Chromatograms of samples of the unhydrolysed material showed that it contained neither nicotinic acid nor glycine.

These experiments establish the identity of the compound formed from nicotinic acid with nicotinuric acid.

Nicotinuric acid synthesis by rat-kidney slices and homogenates

When slices of rat-kidney cortex were incubated in Krebs–Ringer phosphate solution (Krebs, 1933), pH 7·4, together with nicotinic acid, glycine and fumarate, nicotinuric acid was formed. In a number of experiments the synthesis averaged 8–10 μmoles/g. wet wt./2 hr. No synthesis occurred when nicotinic acid was omitted, but in the absence of glycine a small amount of nicotinuric acid was formed. Nicotinuric acid was absent from incubation mixtures stopped at zero time.

Kidney homogenates prepared in 0·154 M-KCl or 0·25 M-sucrose also synthesized nicotinuric acid in the presence of nicotinic acid, glycine, fumarate and ATP (Table 1). The amounts formed (1·5–2·0 μmoles/g. wet wt./2 hr.) were rather small compared with those formed by slices, but when potassium EDTA was added to the homogenate (5 mM final concentration in the homogenate) the activity was increased to a value of 10–15 μmoles/g. wet wt./2 hr., which is somewhat higher than that of slices. EDTA was therefore added to the homogenizing fluid in all subsequent experiments.

The requirements for nicotinuric acid synthesis by homogenates were similar whether or not the homogenate contained EDTA (Table 1). Only nicotinic acid was absolutely required for the reaction, but glycine, fumarate and ATP were all necessary for maximum synthesis.

Fractionation of homogenates. The results of a typical experiment, in which the distribution of the ability to form nicotinuric acid under the standard incubation conditions between different subcellular fractions of rat-kidney homogenate was examined, are shown in Table 2. The nuclear and mitochondrial fractions were found to be approximately equally active but no synthesis was observed in the microsome and soluble fractions either together (as in Table 2) or separately. The summed activities of the individual fractions represented 96 % recovery of

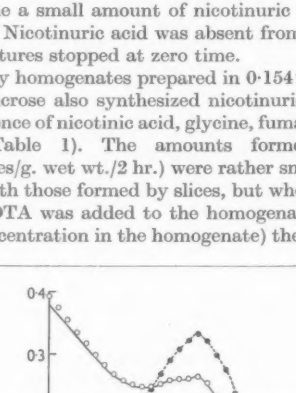


Fig. 1. Comparison of the spectra of synthetic nicotinuric acid and the nicotinic acid metabolite in water and N-HCl. Experimentally determined values for the nicotinic acid metabolite have been multiplied by a factor calculated to give the solutions of the metabolite and of synthetic nicotinuric acid the same extinction at 262 mμ in the same solvent. ——, Spectrum of synthetic nicotinuric acid in water; ○, nicotinic acid metabolite in water; - - - -, synthetic nicotinuric acid in N-HCl; ●, nicotinic acid metabolite in N-HCl.

Table 1. *Nicotinuric acid synthesis by rat-kidney homogenate*

Homogenate A was prepared in 9 vol. of 0·154 M-KCl homogenate B in 8·5 vol. of 0·154 M-KCl + 0·5 vol. of 0·1 M-potassium EDTA, pH 7·4. Incubation conditions are given in the text. A portion (0·05 ml.) of 0·1 M-potassium EDTA, pH 7·4, was added to the incubation mixture where indicated.

Composition of incubation mixture	Nicotinuric acid formed (μmoles/g. wet wt. of tissue/2 hr.) Homogenate A	Homogenate B
Complete	1·7	10·3
Nicotinic acid and glycine omitted	0	0
Nicotinic acid omitted	0	0
Glycine omitted	0·2	1·1
Fumarate omitted	0·7	2·3
ATP omitted	—	6·0
Complete, EDTA added	11·6	—

Table 2. *Nicotinuric acid synthesis in tissue fractions*

Rat-kidney homogenate was fractionated and incubations were performed as described in the text.

Tissue fraction	Nicotinuric acid formed (μmoles/g. wet wt. of tissue/2 hr.)	(μmoles/mg. of total N/2 hr.)
Whole homogenate	11·6	0·59
Nuclear fraction	5·95	2·09
Mitochondrial fraction	5·15	1·85
Microsome plus soluble fraction	0	0
Nuclear + mitochondrial fractions	11·2	—
Mitochondrial + microsome plus soluble fractions	5·2	—

the activity of the whole homogenate. The amounts of nicotinuric acid formed when the fractions were combined agreed well with the sum of their separate activities, and when samples of the nuclear and mitochondrial fractions were combined the observed synthesis was 97 % of that in the whole homogenate.

In other fractionation experiments 90–110 % of the activity of the homogenate was recovered in the nuclear and mitochondrial fractions, but although the total activity of these fractions was about the same in all experiments, its quantitative distribution between them was rather variable. The ratio of the amount of nicotinuric acid formed per gram of tissue by the nuclear fraction to that formed by the mitochondrial fraction varied between 0·85 and 1·7. A rather similar variation was also found in the amounts of total N per gram of tissue in each fraction, and the activities of each fraction calculated per milligram of total N were much more constant from one experiment to another than those per gram of tissue. In all experiments, including those where only one fraction was tested, the nuclear fraction formed 2·0–2·5 μmoles of nicotinuric acid/mg. of total N/2 hr., whereas the mitochondrial fraction normally formed 1·8–2·5 μmoles/mg. of total N/2 hr., with occasional higher values. In contrast to its stimulating effect when added to homogenates, EDTA was found to inhibit the activity of nuclear and mitochondrial suspensions by about one-third when added to the incubation mixtures at a final concentration of 1·6 mM.

It is possible that the microsome and soluble fractions might fail to synthesize nicotinuric acid because of the rapid hydrolysis of the ATP initially present in the incubation mixtures. Experiments were therefore performed with these fractions in which two methods were used to ensure an adequate constant supply of ATP. First, potassium phosphoglycerate and an enzyme fraction from rabbit muscle (Ratner & Pappas, 1949) were added to the incubation mixture. The muscle fraction contains the enzymes of the glycolytic sequence which convert phosphoglycerate into pyruvate with the concomitant rephosphorylation of adenosine diphosphate to ATP. Neither the microsome fraction nor the soluble fraction formed any nicotinuric acid in the presence of this ATP-regenerating system. Secondly, a sample of mitochondrial suspension was added to the incubation mixture under conditions suitable for oxidative phosphorylation. In such experiments no increase in nicotinuric acid formation over that found with the mitochondrial suspension alone was obtained with either the microsome or the soluble fraction.

Nicotinuric acid synthesis in the nuclear fraction. The nuclear fraction isolated in the manner used here is known to contain whole cells and mitochondria in addition to the nuclei (de Duve & Berthet, 1954), and it seemed possible that the nicotinuric acid synthesis observed in the nuclear fraction might be due to these contaminants rather than to the nuclei.

The formation of nicotinuric acid by the nuclear fraction was shown to be absolutely dependent upon the addition of nicotinic acid, glycine, fumarate and ATP (Table 3). An absolute requirement for all these substances was not found in slices or even in homogenates (Table 1), making it unlikely that nicotinuric acid synthesis in the nuclear fraction was brought about by whole cells.

The succinoxidase activity of the nuclear fraction was used to obtain an estimate of the number of mitochondria in this fraction, since succinoxidase is believed to be present only in mitochondria (Dounce, 1955; Schneider & Hogeboom, 1956). Succinoxidase in the nuclear and mitochondrial fractions was assayed by the method of Schneider & Potter (1943) and nicotinuric acid formation by samples of the same suspensions was measured simultaneously. The results of such an experiment are shown in Table 4. It was found that the nuclear fraction derived from 1 g. of kidney had an activity equivalent to 80 % of the succinoxidase activity of the mitochondrial fraction, whereas nicotinuric

Table 3. *Nicotinuric acid synthesis in the nuclear fraction*

The nuclear fraction of rat kidney was sedimented and washed as described in the text and the final pellet suspended in a volume of 0·154 M-KCl equal to one-fifth of that of the homogenate used. Incubation conditions are given in the text.

Composition of incubation mixture	Nicotinuric acid formed (μmoles/mg. of total N/2 hr.)
Stopped at zero time	0
Complete	2·37
Nicotinic acid omitted	0
Glycine omitted	0
Fumarate omitted	0·04
ATP omitted	0·02

Table 4. *Nicotinuric acid synthesis and succinoxidase in the nuclear and mitochondrial fractions*

Activities were measured as described in the text and are given per gram wet weight of tissue. Figures in parentheses are activities per milligram of total N.

Fraction	Nicotinuric acid synthesis (μmoles/2 hr.)	Succinoxidase (O_2 uptake in μl./hr.)
Whole homogenate	11·0 (0·58)	—
Nuclear fraction	7·15 (2·28)	5800 (1850)
Mitochondrial fraction	4·25 (1·85)	7260 (3160)
Ratio: Nuclear fraction / Mitochondrial fraction	1·68	0·80

acid synthesis in the nuclear fraction was 168% of that in the mitochondrial fraction. Assuming that mitochondria isolated in the mitochondrial and nuclear fractions have the same activity, the mitochondria present in the nuclear fraction synthesized 3·4 μmoles of nicotinuric acid/g. of kidney/2 hr., whereas the nuclei formed 3·75 μmoles/g. of kidney/2 hr.

Nicotinuric acid synthesis by mitochondrial suspensions

Requirements for synthesis. Nicotinuric acid was synthesized by mitochondrial suspensions in the presence of nicotinic acid, glycine, fumarate and ATP. If any of these components were omitted from the incubation mixture, no nicotinuric acid was formed and none was present in the mixture stopped at zero time (Table 5).

Table 5. *Nicotinuric acid synthesis by rat-kidney mitochondria*

Incubation conditions are given in the text.

Composition of incubation mixture	Nicotinuric acid formed (μmoles/mg. of total N/2 hr.)
Stopped at zero time	0
Complete	2·40
Nicotinic acid omitted	0
Glycine omitted	0
Fumarate omitted	0
ATP omitted	0·01

Table 6. *Effect of period of incubation upon nicotinuric acid synthesis and oxygen uptake of rat-kidney mitochondria*

Incubation conditions were as given in the text, except that 0·3 ml. of 0·1 M-potassium fumarate was used. Incubations were performed in Warburg manometers. Potassium nicotinate and glycine were placed in the side arm and tipped after equilibration.

Time of incubation (min.)	Uptake of O_2 (μl./mg. of total N)	Nicotinuric acid formed (μmoles/mg. of total N)	Uptake of O_2 (μl./μmole of nicotinuric acid formed)
0	0	0	0
30	222	1·10	202
60	426	2·13	200
90	575	2·71	212
120	630	3·07	205
150	610	2·90	210
180	662	2·70	245

Table 7. *Effect of fumarate concentration upon nicotinuric acid synthesis and uptake of oxygen of rat-kidney mitochondria*

Incubations were performed in Warburg manometers under the conditions given in the text. Potassium nicotinate and glycine were placed in the side arm and tipped after equilibration.

Concn. of fumarate (M)	Uptake of O_2 (μl./mg. of total N/2 hr.)	Nicotinuric acid formed (μmoles/mg. of total N/2 hr.)	Uptake of O_2 (μl./μmole of nicotinuric acid formed)
0	0	0	—
0·005	440	1·63	270
0·01	625	2·20	284
0·02	780	2·40	324

Relation of synthesis to energy supply. Table 6 shows the nicotinuric acid synthesis and the oxygen uptake by mitochondrial suspensions after different periods of incubation. Both proceeded linearly for about 90 min., and after 2 hr. the oxygen uptake continued to rise only very slowly and no further nicotinuric acid synthesis was observed. The oxygen uptake per μmole of nicotinuric acid formed was constant irrespective of the length of incubation (Table 6). When the concentration of fumarate was varied the oxygen uptake per μmole of nicotinuric acid formed was again constant and was independent of the fumarate concentration (Table 7).

Nicotinuric acid synthesis was inhibited by 2:4-dinitrophenol at concentrations of 20 μM to 0·5 mM, concentrations which uncouple oxidative phosphorylation. No synthesis occurred when the incubation was performed anaerobically.

Effect of concentration of substrates. The effect upon nicotinuric acid synthesis of varying the nicotinic acid concentration is shown in Fig. 2. Maximum synthesis was obtained at a concentration of 3 mM, but at higher nicotinic acid concentrations the synthesis was inhibited and at 0·04 M-nicotinic acid it was half of that at 3 mM. At low concentrations a large proportion of the nicotinic acid was converted into nicotinuric acid; in the experiment illustrated in Fig. 2, 71% conversion was observed at 1 mM and in other experiments up to 95% conversion was found at lower concentrations.

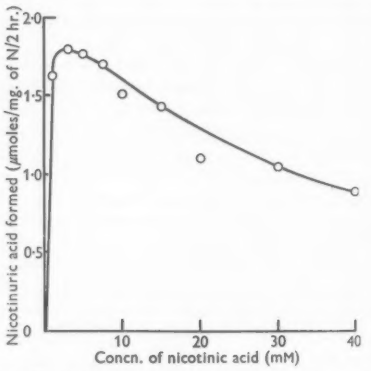


Fig. 2. Effect of nicotinic acid concentration upon nicotinuric acid synthesis by rat-kidney mitochondria. Incubation conditions are given in the text.

Fig. 3 shows the nicotinuric acid synthesis at different glycine concentrations. Maximum synthesis was obtained at about 0·04 M-glycine and no inhibition was observed at concentrations up to 0·08 M. The K_m of the system towards glycine was about 7·5 mM.

The effect of different ATP concentrations is illustrated in Fig. 4. In this experiment, maximum synthesis was obtained with a concentration of 1 mM, and at higher concentrations inhibition occurred, the synthesis at 2 mM-ATP being 65 % of the maximum. The cause of this inhibition is not known, but it seems possible that it was due to an impurity in the ATP preparations rather than to ATP itself since optimum synthesis was obtained at slightly different ATP concentrations with different ATP preparations.

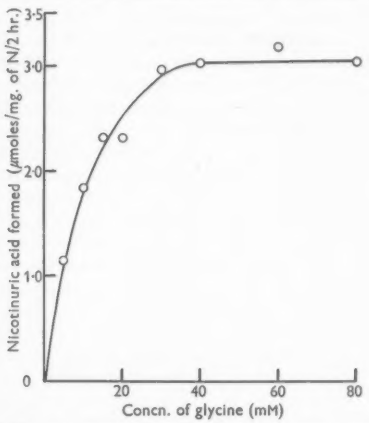


Fig. 3. Effect of glycine concentration upon nicotinuric acid synthesis by rat-kidney mitochondria. Incubation conditions are given in the text.

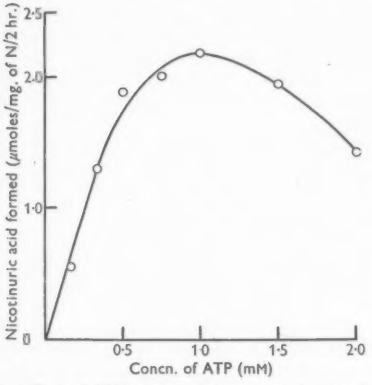


Fig. 4. Effect of ATP concentration upon nicotinuric acid synthesis by rat-kidney mitochondria. Incubation conditions are given in the text.

Inhibitors of nicotinuric acid synthesis. Nicotinuric acid formation by mitochondrial suspensions was inhibited by potassium fluoride, 82 % inhibition being observed at a concentration of 10 mM (Table 8). The effect of magnesium concentration is also shown in Table 8. In the absence of fluoride, a 39 % decrease in synthesis was found when the magnesium concentration was raised from 1 to 5 mM, but when fluoride was present the synthesis was unaffected by changing the magnesium concentration.

The formation of nicotinuric acid was strongly inhibited by benzoic acid, inhibition being 14 % at 0·1 mM-, 33 % at 0·3 mM- and 98 % at 1 mM-benzoic acid. The degree of inhibition caused by 0·1 mM-benzoic acid was unaffected by variation of the nicotinic acid concentration over a fourfold range, suggesting that the inhibition was non-competitive. Benzoic acid and hippuric acid were shown not to interfere with the estimation of nicotinuric acid.

Effect of ammonia. The addition of ammonium chloride to the incubation mixtures at concentrations of 3 mM to 0·03 M had no effect upon the nicotinuric acid synthesis. No nicotinamide was formed from nicotinic acid and ammonia by these preparations, either in the presence or in the absence of glycine.

DISCUSSION

The experiments described show that rat kidney and liver contain an enzyme system which forms nicotinuric acid from nicotinic acid and glycine. With slices and homogenates some nicotinuric acid was formed from nicotinic acid in the absence of added glycine, showing that the tissues contain sufficient free glycine for nicotinuric acid synthesis to occur *in vivo* so long as nicotinic acid is available. These observations are a further proof of the ability of the rat to form nicotinuric acid, and remove any remaining doubt on this point (Ellinger & Abdel Kader, 1949).

Table 8. *Effect of magnesium and fluoride ions upon nicotinuric acid synthesis by rat-kidney mitochondria*

Incubation conditions are given in the text.

Concn. of KF (mM)	Nicotinuric acid formed (μmole/mg. of total N/2 hr.) With 1 mM-$MgCl_2$	With 5 mM-$MgCl_2$
0	1·97	1·19
1	1·54	—
5	0·61	0·60
10	0·36	0·33

Distribution of nicotinuric acid synthesis between tissue fractions. The results show that the nicotinuric acid-synthesizing ability of the homogenate is localized in the nuclear and mitochondrial fractions, being approximately equally divided between the two. None was found in the microsome or soluble fractions even when an external source of energy was added. This distribution differs from those found for other similar reactions forming peptide-like bonds. For example, Kielley & Schneider (1950) showed that in mouse liver *p*-aminohippuric acid synthesis is located almost entirely in the mitochondrial fraction, whereas taurocholic acid synthesis in guinea-pig liver occurs in the microsomes but not in the mitochondria (Elliott, 1956*a*, *b*). These differences may be due to different enzymes being involved in the several reactions or may merely reflect variations in the distribution of the same enzymes in different tissues.

The synthetic activity of the nuclear fraction is of particular interest since the only energy-requiring reaction of known mechanism previously shown to occur in this fraction is the synthesis of diphosphopyridine nucleotide from ATP and nicotinamide mononucleotide, which is almost exclusively located there in rat liver (Hogeboom & Schneider, 1952).

The evidence that some of the activity of the nuclear fraction was due to the nuclei themselves and some to contaminating mitochondria is based upon the differences in the distribution of succinoxidase and nicotinuric acid-synthesizing activities between the nuclear and mitochondrial fractions. The interpretation of this experiment is based upon certain assumptions. First, that the succinoxidase was, in fact, all in the mitochondria, since its localization in these structures has been demonstrated only in liver. Secondly, it was assumed that a given number of mitochondria had the same succinoxidase and nicotinuric acid-synthesizing activities whether they were isolated in the nuclear or the mitochondrial fraction; that is to say that these activities were uninfluenced by the other components of the nuclear fraction.

It appeared possible that the second assumption particularly might not be true and that the nuclei might exert some protective action on the mitochondria in the nuclear fraction, for example by binding an inhibitor, which would result in these mitochondria being more active, unit for unit, than those in the mitochondrial fraction. This suggestion was, however, not borne out by the evidence. First, the possibility that the nuclei act by binding an inhibitory metal ion appeared unlikely in view of the observation that with both the nuclear and mitochondrial fractions the addition of EDTA to the incubation mixture caused an inhibition of nicotinuric acid synthesis. Secondly, it would be expected that any protective action of the nuclei would be fully effective in a homogenate, but that when the nuclei were separated from the other components of the homogenate by fractionation, the sum of the activities of the individual fractions would be less than that of the whole homogenate. In fact, 90–110% of the activity of the whole homogenate was always recovered in the fractions. Furthermore, it would also be expected that the addition of a sample of the nuclear fraction to the mitochondrial fraction would lead to a greater synthesis than that calculated from their separate activities. No such effect was ever observed. These two last-named facts particularly make the possibility of the nuclei having a protective action on the mitochondria appear most unlikely.

The simplest explanation of all these observations seems to be that both the nuclei and the mitochondria possess the ability to synthesize nicotinuric acid, but a definite answer to this question must await the isolation of uncontaminated rat-kidney nuclei.

The variable distribution of the synthetic activity and of total N between the nuclear and mitochondrial fractions was probably due to the sedimentation of variable numbers of mitochondria with the nuclear fraction. Hers, Berthet, Berthet & de Duve (1951) have noted that kidney homogenates are particularly liable to clumping of the particles, which causes the mitochondria to sediment with the nuclear fraction, on account of their tendency to form acid rapidly, or, if buffer is added to counteract the lowering of pH, because of the increased ionic strength of the homogenizing medium. In these experiments, the homogenizing medium contained a small amount of buffer (5 mM-potassium EDTA) and so clumping would probably occur for both reasons. The degree of clumping would presumably depend upon the precise treatment of the homogenate in question, particularly upon the time of standing between homogenization and centrifuging, and might vary appreciably from one experiment to another, leading to differences in the numbers of mitochondria sedimented in the nuclear fraction.

Nicotinuric acid synthesis in mitochondrial suspensions. The close relationship between nicotinuric acid synthesis and oxidation is shown by the constancy of the oxygen uptake per μmole of nicotinuric acid formed, in spite of variations in the period of incubation and the fumarate concentration, and by the absence of any synthesis under anaerobic conditions. The inhibition by 2:4-dinitrophenol shows that, with mitochondrial suspensions, oxidative phosphorylation is required for the synthesis. In these suspensions, oxidative phosphorylation cannot be adequately replaced by added ATP, presumably owing to the rapid hydrolysis of the latter.

A possible explanation of the non-competitive

inhibition of nicotinuric acid formation by benzoic acid has been suggested by the observation of Schachter & Taggart (1954) that the condensation of various acyl-coenzyme A compounds with glycine to form the corresponding acyl-glycine compounds is inhibited non-competitively by hippuric acid. If an enzyme with similar properties participates in nicotinuric acid synthesis, it is possible that benzoic acid was first converted into hippuric acid, a conversion which these mitochondrial suspensions were able to perform, and that hippuric acid was the true inhibitor of nicotinuric acid synthesis.

The present investigation of nicotinuric acid synthesis was undertaken partly in the hope that an activated form of nicotinic acid might be found which reacted not only with glycine to form nicotinuric acid but also with ammonia to form nicotinamide. The absence of any effect of ammonia upon nicotinuric acid synthesis and the failure to find any nicotinamide synthesis when glycine was replaced by ammonia show that this is not the case in rat-kidney mitochondria.

The mechanism of nicotinuric acid synthesis is discussed in the next paper (Jones, 1959*a*).

SUMMARY

1. Slices and homogenates of rat kidney and liver have been shown to synthesize nicotinuric acid from nicotinic acid and glycine. Nicotinuric acid was identified chromatographically, by its spectrum and by hydrolysis to nicotinic acid and glycine. The synthesis in homogenates was stimulated by fumarate and adenosine triphosphate.

2. The synthetic activity of rat-kidney homogenate was located entirely in the nuclear and mitochondrial fractions. None was found in the microsome and soluble fractions even when an energy-supplying system was added. About half the activity of the nuclear fraction appeared to be due to the nuclei, the remainder being caused by contaminating mitochondria.

3. Nicotinuric acid synthesis by rat-kidney mitochondria was completely dependent upon added nicotinic acid, glycine, fumarate and adenosine triphosphate. The synthesis was inhibited by 2:4-dinitrophenol, benzoic acid and potassium fluoride. The properties of the synthetic system in mitochondria are described.

4. Nicotinamide was not synthesized by the enzyme system which synthesized nicotinuric acid.

We wish to thank Professor Sir Hans A. Krebs, F.R.S., for his valuable suggestions and for his interest in this work. One of the authors (K.M.J.) is indebted to the Christopher Welch Trust, to St John's College, Oxford, and to the Medical Research Council for the award of scholarships held during the course of this work. The work was aided by a grant to the Department from the Rockefeller Foundation.

REFERENCES

Ackermann, D. (1912). *Z. Biol.* **59**, 17.
Consden, R., Gordon, A. H. & Martin, A. J. P. (1947). *Biochem. J.* **41**, 590.
de Duve, C. & Berthet, J. (1954). *Int. Rev. Cytol.* **3**, 225.
Dounce, A. L. (1955). In *The Nucleic Acids*, vol. 2, p. 93. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press Inc.
Ellinger, P. & Abdel Kader, M. M. (1949). *Biochem. J.* **44**, 77.
Elliott, W. H. (1956*a*). *Biochem. J.* **62**, 427.
Elliott, W. H. (1956*b*). *Biochem. J.* **62**, 433.
Hers, H. G., Berthet, J., Berthet, L. & de Duve, C. (1951). *Bull. Soc. Chim. biol., Paris*, **33**, 21.
Hogeboom, G. H. & Schneider, W. C. (1952). *J. biol. Chem.* **197**, 611.
Huff, J. W. & Perlzweig, W. A. (1942). *J. biol. Chem.* **142**, 401.
Johnson, B. C. & Lin, P.-H. (1953). *J. Amer. chem. Soc.* **75**, 2971.
Jones, K. M. (1959*a*). *Biochem. J.* **73**, 714.
Jones, K. M. (1959*b*). *Biochem. J.* **73**, 719.
Jones, K. M. & Elliott, W. H. (1954). *Biochim. biophys. Acta*, **14**, 586.
Kerr, S. E. (1941). *J. biol. Chem.* **139**, 121.
Kielley, R. K. & Schneider, W. C. (1950). *J. biol. Chem.* **185**, 869.
Kodicek, E. & Reddi, K. K. (1951). *Nature, Lond.*, **168**, 475.
Krebs, H. A. (1933). *Hoppe-Seyl. Z.* **217**, 191.
Leifer, E., Roth, L. J., Hogness, D. S. & Corson, M. H. (1951). *J. biol. Chem.* **190**, 595.
Levine, R. & Snead, J. K. (1951). *J. Amer. chem. Soc.* **73**, 5614.
Lin, P.-H. & Johnson, B. C. (1953). *J. Amer. chem. Soc.* **75**, 2974.
Markham, R. & Smith, J. D. (1949). *Biochem. J.* **45**, 294.
Melnick, D., Robinson, W. D. & Field, H., jun. (1940). *J. biol. Chem.* **136**, 145.
Needham, D. M. (1942). *Biochem. J.* **36**, 113.
Potter, V. R. & Elvehjem, C. A. (1936). *J. biol. Chem.* **114**, 495.
Ratner, S. & Pappas, A. (1949). *J. biol. Chem.* **179**, 1183.
Reddi, K. K. & Kodicek, E. (1953). *Biochem. J.* **53**, 286.
Rohrlich, M. (1951). *Arch. Pharm., Berl.*, **284**, 6.
Schachter, D. & Taggart, J. V. (1954). *J. biol. Chem.* **211**, 271.
Schneider, W. C. (1948). *J. biol. Chem.* **176**, 259.
Schneider, W. C. & Hogeboom, G. H. (1956). *Annu. Rev. Biochem.* **25**, 201.
Schneider, W. C. & Potter, V. R. (1943). *J. biol. Chem.* **149**, 217.

The Mechanism of Nicotinuric Acid Synthesis

By K. M. JONES
Department of Biochemistry, University of Oxford

(*Received* 14 *April* 1959)

In the previous paper (Jones & Elliott, 1959) it was shown that rat-kidney mitochondria are able to synthesize nicotinuric acid from nicotinic acid and glycine in the presence of fumarate and adenosine triphosphate (ATP). These experiments showed that nicotinuric acid synthesis is dependent upon a suitable source of energy, but did not give any other indications of the mechanism of the reaction.

Although the mechanism of nicotinuric acid synthesis has not previously been investigated, the formation of other acyl-glycine compounds, particularly hippuric acid and *p*-aminohippuric acid, has been extensively studied. The work of Chantrenne (1951) with extracts of acetone-dried rat-liver powder first showed that coenzyme A (CoA) was required for hippuric acid synthesis. Subsequently, Schachter & Taggart (1953) showed that hippuric acid synthesis proceeded in two steps, with benzoyl-CoA as an intermediate:

$$\text{Benzoic acid} + \text{ATP} + \text{CoA} \rightarrow \text{benzoyl-CoA} + \text{adenosine 5'-phosphate} + \text{inorganic pyrophosphate} \quad (1)$$

$$\text{Benzoyl-CoA} + \text{glycine} \rightarrow \text{hippuric acid} + \text{CoA}. \quad (2)$$

Their enzyme preparation was able to form hippuric acid from chemically synthesized benzoyl-CoA and glycine as well as from benzoic acid, glycine, adenosine triphosphate and CoA. In a subsequent paper (Schachter & Taggart, 1954), the specificity of the enzyme catalysing the activation of benzoic acid (reaction 1) was examined by measuring the formation of inorganic pyrophosphate when a preparation from acetone-dried ox-liver mitochondria was incubated with various acids together with adenosine triphosphate and CoA. The specificity of the enzyme catalysing reaction 2 (called glycine-*N*-acylase) for the acyl-CoA reactant was investigated by adding glycine to the incubation mixture and measuring the increase in pyrophosphate release over that brought about by reaction 1. It was shown with butyric acid and valeric acid that the extra pyrophosphate formation caused by adding glycine was equal in amount to the butyrylglycine or valerylglycine formed. In this way both enzymes were shown to have a wide specificity among aliphatic and aromatic acids.

Among the acids tested was nicotinic acid, which gave a pyrophosphate release of 0·13 μmole without glycine and 0·15 μmole with glycine. Thus it appears that the activating enzyme in this preparation was able to form nicotinoyl-CoA from nicotinic acid, adenosine triphosphate and CoA, but it is by no means clear from the very small increase in pyrophosphate release caused by the addition of glycine whether glycine-*N*-acylase will bring about the synthesis of nicotinuric acid from nicotinoyl-CoA and glycine.

In this paper it is shown that an enzyme preparation similar to that used by Schachter & Taggart (1954) in their specificity studies can, in fact, synthesize nicotinuric acid, and its use in investigating the mechanism of this reaction is described.

EXPERIMENTAL

Materials

For the preparation and purification of materials other than those given below, see Jones & Elliott (1959).

Nicotinoylhydroxamic acid. A solution of nicotinoylhydroxamic acid suitable for use as a chromatographic marker was prepared as follows. Hydroxylamine hydrochloride (0·14 g.; 2 m-moles) and 0·1 g. (4 m-moles) of metallic sodium were separately dissolved in the minimum volumes of dry methanol. These solutions were mixed and 0·14 g. (1 m-mole) of methyl nicotinate dissolved in 2 ml. of dry methanol was added. The mixture was left to stand at room temperature for 6 hr. and then neutralized to pH 6·8 with methanolic HCl. The NaCl was filtered off and washed with methanol. The filtrate was made up to 20 ml. with methanol and stored at $-15°$.

Coenzyme A. Preparations from pig liver containing 10–13% of coenzyme A (CoA) were made by the method of Elliott (1956*a*). Solutions were prepared in water and neutralized with KOH. A sample of 70% pure CoA was kindly supplied by Dr B. C. Loughman. CoA was assayed by the method of Kaplan & Lipmann (1948), or that of Stadtman & Kornberg (1953), with transacetylase from *Escherichia coli*, kindly prepared by Dr J. Lascelles. Quantities of CoA are expressed in the units of Kaplan & Lipmann (1948).

Hydroxylamine. Hydroxylamine solutions of low salt content (salt-free hydroxylamine) were prepared by the method of Beinert *et al.* (1953). A saturated methanolic solution of hydroxylamine hydrochloride was neutralized to pH 7·2 with strong methanolic KOH. The precipitate of KCl was removed by filtration and the resulting solution was evaporated to a small volume by distillation under reduced pressure. The hydroxylamine content was estimated by titration with iodine or HCl and the solution adjusted to 2 M by the addition of water. This solution was stored at $-15°$ for a maximum of 7 days and adjusted to pH 8·0 immediately before use.

2-Amino-2-hydroxymethylpropane-1:3-diol buffer. This buffer (tris) was prepared by neutralizing solutions of the free base with HCl.

Methods

Preparation of the enzyme solution. The enzyme solution used in these experiments was prepared by a modification of the method of Schachter & Taggart (1954).

An acetone-dried powder of ox-liver mitochondria was prepared as described by Mahler, Wakil & Bock (1953). About 30 g. of powder was obtained/kg. of liver used. The acetone-dried powder was extracted by continuous stirring for 30 min. with 10 vol. of 0·02 M-phosphate buffer, pH 7·5. The mixture was centrifuged and the supernatant decanted. Solid $(NH_4)_2SO_4$ (24·7 g.) was then added to each 100 ml. of the supernatant (35% saturation). The solution was centrifuged and a further 10·6 g. of $(NH_4)_2SO_4$ added to each 100 ml. of the supernatant (50% saturation). The precipitate was collected by centrifuging, made into a paste with a little water and dialysed overnight against two changes of 0·02 M-$KHCO_3$. From 64 g. of acetone-dried powder the yield was 120 ml. of enzyme solution, which was kept at −15° for 3 months with only a small decrease in activity.

Dowex-treated enzyme. A sample of the enzyme solution was mixed with an equal volume of semi-dry Dowex-1 (10% cross-linked) in the chloride form and stirred for 15 min. The mixture was centrifuged and the supernatant filtered to remove particles of resin.

Measurement of activity. The standard incubation mixture used for studying nicotinuric acid synthesis had the following composition: 0·2 ml. of 0·25 M-tris buffer, pH 8·0; 0·03 ml. of 0·1 M-$MgCl_2$; 0·03 ml. of 0·1 M-adenosine triphosphate (ATP); 0·05 ml. of 0·1 M-potassium nicotinate; 0·2 ml. of 0·3 M-glycine; 40 units of CoA; 0·25 ml. of enzyme solution; water to give a total volume of 1 ml. Incubations were performed in centrifuge tubes at 38° for 30 min. After incubation, the mixtures were deproteinized by placing in a boiling-water bath and centrifuging. Samples (0·25 ml.) of the supernatants were taken for the estimation of nicotinuric acid by the method of Jones (1959).

The standard incubation conditions used for the investigation of nicotinoylhydroxamic acid formation were as given above except that 0·25 ml. of 2 M-hydroxylamine (salt-free) was added in place of glycine. After incubation, hydroxamic acid was estimated as described below. Nicotinoylhydroxamic acid synthesis was determined by measuring the difference in hydroxamic acid formation between incubations with and without nicotinic acid.

Estimation of hydroxamic acids. Hydroxamic acids were estimated by a modification of the method used by Kornberg & Pricer (1953) for soluble hydroxamic acids. Incubation mixtures were deproteinized by adding 1 ml. of 7% (v/v) $HClO_4$ and centrifuging. The supernatants were poured off into a mixture of 0·6 ml. of 7% (v/v) $HClO_4$ and 0·4 ml. of 10% (w/v) $FeCl_3,6H_2O$ in 0·2 N-HCl, and water was then added to bring the volume to 4 ml. Extinctions were measured at 540 mμ against a reagent blank.

A standard curve for nicotinoylhydroxamic acid was obtained by the method of Elliott (1956*a*), modified somewhat because of differences in the methods of hydroxamic acid estimation used. Suitable volumes of a solution of methyl nicotinate in methanol (2 μmoles/ml.) were mixed with 1 ml. of alkaline hydroxylamine solution, prepared by mixing equal volumes of 2 M-hydroxylamine hydrochloride and 2·5 N-NaOH, and methanol was added to bring the total volume to 1·5 ml. After the solutions had been set aside for 15 min. at room temperature, 0·1 ml. of 6 N-HCl was added to each tube, followed by 1·6 ml. of 7% (v/v) $HClO_4$, 0·4 ml. of 10% (w/v) $FeCl_3,6H_2O$ in 0·2 N-HCl and 0·4 ml. of water, making a final volume of 4 ml. Extinctions were read at 540 mμ against a blank solution treated as above but containing no methyl nicotinate. The extinctions obtained were proportional to the amount of methyl nicotinate added over the range 0–1 μmole.

Chromatographic methods. The chromatographic separation of tertiary nicotinic acid compounds and their detection by treatment with cyanogen bromide and *p*-aminobenzoic acid have been described by Jones & Elliott (1959).

On chromatography in butan-1-ol–water synthetic nicotinoylhydroxamic acid gave a somewhat elongated spot with an R_F value slightly less than that of nicotinamide. On treatment with cyanogen bromide and *p*-aminobenzoic acid it gave a yellow with a slight brownish tinge immediately on spraying, turning to canary yellow when dry; these colours allowed the nicotinoylhydroxamic acid spot to be clearly distinguished from that of nicotinamide. It also gave the brownish-purple characteristic of hydroxamic acids when sprayed with 10% (w/v) $FeCl_3,6H_2O$ in 0·2 N-HCl.

RESULTS

Synthesis of nicotinuric acid by the enzyme preparation. The enzyme preparation synthesized nicotinuric acid when incubated with nicotinic acid, glycine, ATP and CoA (Table 1). Nicotinic acid, glycine and ATP were all absolutely required for the synthesis. Added CoA, however, was not necessary for nicotinuric acid formation, but when 40 units of CoA were added to the incubation mixture the synthesis was 50% greater than in its absence.

The time course of nicotinuric acid formation is shown in Fig. 1. The synthesis proceeded linearly for 30 min. and then fell off abruptly.

The enzyme preparation was unable to form nicotinamide from nicotinic acid when glycine was replaced by NH_4Cl (3–30 mM, final concentration). This confirms results obtained with whole rat-kidney mitochondria (Jones & Elliott, 1959), indicating that nicotinamide is not synthesized by a route similar to that for nicotinuric acid formation.

Table 1. *Nicotinuric acid synthesis by the enzyme preparation*

Incubation conditions are given in the text.

Composition of incubation mixture	Nicotinuric acid formed (μmole/30 min.)
Complete mixture	1·13
Nicotinic acid omitted	0·0
Glycine omitted	0·01
ATP omitted	0·0
CoA omitted	0·75

Hydroxamic acid formation by the enzyme preparation. When glycine was replaced in the incubation mixture by salt-free hydroxylamine solution, the enzyme preparation formed a hydroxamic acid (Table 2). The values in Table 2 have been corrected for the hydroxamic acid-reacting material present in an incubation mixture stopped at zero time. Hydroxamic acid formation was completely dependent only upon ATP and hydroxylamine. In the absence of nicotinic acid a small hydroxamic acid blank was obtained, presumably due to traces of carboxylic acids in the enzyme solution or the CoA preparation. On the addition of nicotinic acid a large increase in hydroxamic acid formation occurred and it was assumed that only this increase represented nicotinoylhydroxamic acid. The identification of this substance with nicotinoylhydroxamic acid is described below. Nicotinoylhydroxamic acid formation occurred without added CoA, but a 47 % stimulation of synthesis was observed when 40 units of CoA were added. Glutathione inhibited nicotinoylhydroxamic acid formation, 23 % inhibition being found with 0·02 M-glutathione.

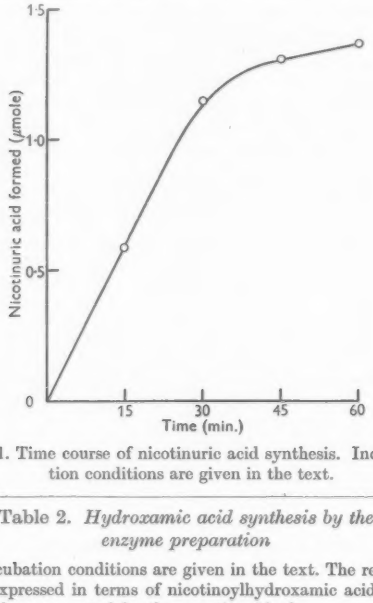


Fig. 1. Time course of nicotinuric acid synthesis. Incubation conditions are given in the text.

Table 2. *Hydroxamic acid synthesis by the enzyme preparation*

Incubation conditions are given in the text. The results are expressed in terms of nicotinoylhydroxamic acid and have been corrected for the zero time blank.

Composition of incubation mixture	Hydroxamic acid formed (μmoles/30 min.)
Complete mixture	0·56
Nicotinic acid omitted	0·05
Hydroxylamine omitted	0·0
ATP omitted	0·01
CoA omitted	0·38

When glycine and hydroxylamine were present in the same incubation mixture, the syntheses of both nicotinuric acid and nicotinoylhydroxamic acid were inhibited by 50 % compared with the amounts obtained with glycine or hydroxylamine singly.

The effect of hydroxylamine concentration on nicotinoylhydroxamic acid synthesis is shown in Fig. 2. Maximum synthesis was obtained with a hydroxylamine concentration of 0·5 M. At 0·75 M a

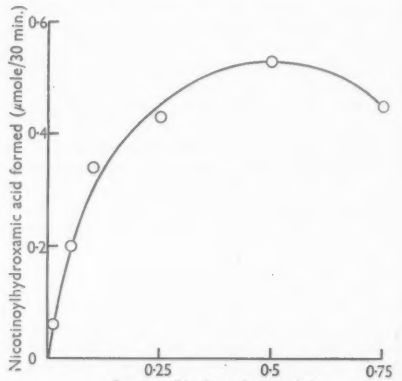


Fig. 2. Effect of hydroxylamine concentration upon nicotinoylhydroxamic acid synthesis. Values have been corrected for blanks obtained at each hydroxylamine concentration in the absence of nicotinic acid. Incubation conditions are given in the text.

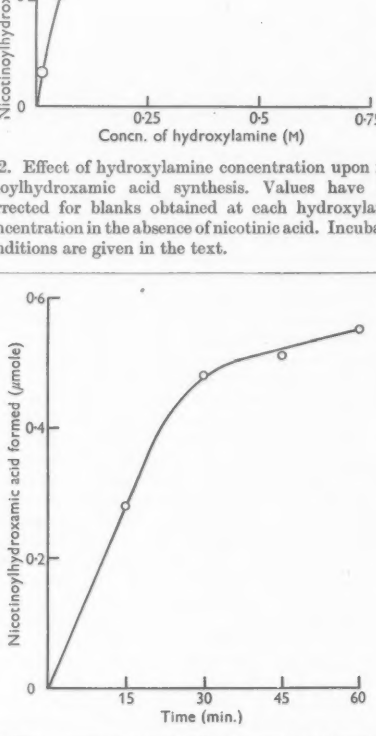


Fig. 3. Time course of nicotinoylhydroxamic acid synthesis. Values have been corrected for blanks obtained at each time in the absence of nicotinic acid. Incubation conditions are given in the text.

small degree of inhibition was observed which may have been due to methanol present in the hydroxylamine solution.

The time course of nicotinoylhydroxamic acid formation was similar to that for nicotinuric acid formation, the rate of synthesis being constant up to about 30 min. and then falling off (Fig. 3).

Identification of the hydroxamic acid formed by the enzyme preparation. In order to identify the enzymically formed hydroxamic acid, mixtures were set up with and without nicotinic acid and, after incubation for 30 min. at 38°, the reaction was stopped by placing the tubes in a boiling-water bath for 45 sec. The mixtures were centrifuged and 0·5 ml. samples of the supernatants together with suitable markers were spotted onto Whatman no. 3 paper. Two such chromatograms were developed in butan-1-ol–water for 12 hr.

One of these chromatograms was sprayed with 10% (w/v) $FeCl_3.6H_2O$ in 0·2N-HCl to detect hydroxamic acids. No hydroxamic acid was found in the incubation mixture without nicotinic acid. In the complete mixture a single hydroxamic acid spot having the same R_F as synthetic nicotinoylhydroxamic acid was present, and this was reinforced when synthetic nicotinoylhydroxamic acid was added to the complete mixture after incubation.

The duplicate chromatogram was treated with cyanogen bromide and *p*-aminobenzoic acid to show up nicotinic acid derivatives. In the complete incubation mixture a spot appeared having the same R_F as the enzymically synthesized hydroxamic acid, showing that this hydroxamic acid was also a nicotinic acid derivative. This spot corresponded, both in position and in the colours given, with synthetic nicotinoylhydroxamic acid, and was reinforced by the synthetic substance. None of the substance was present in the incubation mixture lacking nicotinic acid.

These experiments establish the identity of the enzymically-formed hydroxamic acid with nicotinoylhydroxamic acid.

Effect of coenzyme A. The presence of added CoA was not necessary for the enzyme preparation to form either nicotinuric acid or nicotinoylhydroxamic acid, but in both cases a stimulation of synthesis of about 50% occurred when it was added at a concentration of 40 units/ml. The effect of various CoA concentrations upon nicotinoylhydroxamic acid synthesis is shown in Table 3; optimum synthesis took place with 30–40 units of CoA added/ml. At higher concentrations CoA inhibited synthesis, so that at 80 units/ml. the activity was only 7% higher than that obtained without added CoA. It is not known whether this inhibition was due to CoA itself or to other substances present in the CoA preparations, which contained only 10–13% of CoA. The effect of added CoA upon nicotinuric acid synthesis is exactly similar to its effect upon nicotinoylhydroxamic acid formation (Table 3).

It seemed from these results that CoA was required for the synthesis of both nicotinuric acid and nicotinoylhydroxamic acid, but that the enzyme preparation contained sufficient of the coenzyme to give considerable activity without any being added to the incubation mixtures. In order to determine more clearly whether CoA was involved in the reactions, the enzyme preparation was treated with Dowex-1 to remove CoA.

The Dowex-treated enzyme formed a small amount of nicotinoylhydroxamic acid in the absence of added CoA, but only about one-fifth of the amount formed by the untreated enzyme (Table 3). On the addition of CoA (40 units/ml.) there was a 260% increase in nicotinoylhydroxamic acid formation. The inhibition at higher CoA concentrations was still found, the activity at 80 units/ml. showing only a 150% increase over that in the absence of CoA, and the Dowex treatment had not greatly affected the optimum concentration of CoA. The synthesis of nicotinuric acid by the Dowex-treated enzyme in the absence of added CoA was likewise much lower than that by the untreated enzyme (Table 3). The addition of 40 units of CoA/ml. caused a 270% increase in nicotinuric acid synthesis, whereas at 80 units/ml. the increase observed was only 164%.

In order to show that the active component in the impure (10–13%) CoA preparations used was, in

Table 3. *Effect of coenzyme A on nicotinoylhydroxamic acid synthesis and nicotinuric acid synthesis*

Incubation conditions are given in the text.

Concn. of CoA (units/ml.)	Nicotinoylhydroxamic acid formed (μmole/30 min.)		Nicotinuric acid formed (μmole/30 min.)	
	Untreated enzyme	Dowex-treated enzyme	Untreated enzyme	Dowex-treated enzyme
0	0·37	0·08	0·75	0·25
20	0·51	0·24	—	—
40	0·53	0·29	1·13	0·92
60	0·44	0·26	—	—
80	0·40	0·20	0·88	0·66

fact, CoA, a more highly purified preparation (about 70% of CoA) was employed. This preparation had the same stimulating effect upon nicotinoylhydroxamic acid synthesis by the Dowex-treated enzyme as the less pure preparation.

Accumulation of an intermediate in nicotinuric acid synthesis. When the Dowex-treated enzyme was incubated with nicotinic acid, ATP and CoA, but without glycine and hydroxylamine, a substance accumulated which reacted non-enzymically with hydroxylamine to form a hydroxamic acid. When glycine was present in the incubations, none of this substance was found. The formation of the substance was estimated as follows. After incubation, the mixtures were adjusted to pH 5·0, and deproteinized by placing the tubes in a boiling-water bath and then centrifuging. The supernatants were neutralized to pH 7·0 and 1 ml. of 2M-hydroxylamine (salt-free) was added to each. The solutions were left to stand at room temperature for 15 min. and their hydroxamic acid contents estimated. Blank values obtained with incubation mixtures containing no nicotinic acid were subtracted from the experimental values. In this way the accumulation of 0·09 μmole of the substance was shown in the incubation performed in the absence of glycine, but none was found when glycine was present in the incubations.

DISCUSSION

The high concentration of hydroxylamine required for maximal nicotinoylhydroxamic acid formation by the enzyme preparation suggests that the hydroxamic acid was formed by a non-enzymic reaction between hydroxylamine and an enzymically formed carboxyl-activated form of nicotinic acid. Since ATP and CoA were both required for the formation of nicotinoylhydroxamic acid, it seems that the activated form of nicotinic acid is nicotinoyl-CoA, and that the overall reaction proceeds in two steps:

$$\text{Nicotinic acid} + \text{ATP} + \text{CoA} \rightarrow \text{Nicotinoyl-CoA} \quad (3)$$

$$\text{Nicotinoyl-CoA} + \text{hydroxylamine} \rightarrow \text{Nicotinoylhydroxamic acid} + \text{CoA}. \quad (4)$$

The results presented also show that nicotinoyl-CoA is an intermediate in nicotinuric acid synthesis, reaction 3 being followed in this case by reaction 5:

$$\text{Nicotinoyl-CoA} + \text{glycine} \rightarrow \text{Nicotinuric acid} + \text{CoA}. \quad (5)$$

The nature of the ATP breakdown accompanying nicotinoyl-CoA formation has not been investigated, but it seems likely that it is the same as has been found with other carboxylic acid-activating reactions of this type, the products being adenosine 5′-phosphate and inorganic pyrophosphate (Lipmann, Jones, Black & Flynn, 1952; Kornberg & Pricer, 1953; Mahler *et al.* 1953; Elliott, 1957).

The mechanism proposed for nicotinuric acid synthesis is the same as that found for hippuric acid synthesis (Schachter & Taggart, 1953, 1954). This type of mechanism for the formation of –CO·NH– bonds, involving condensation of an acyl-CoA compound with an amino group, is widespread though by no means universal, and is also found, for example, in the acetylation of aromatic amines (Stadtman, Novelli & Lipmann, 1951; Lynen, Reichert & Rueff, 1951) and in taurocholic acid synthesis (Elliott, 1956*a*, *b*). It seems probable that, as has been proved in the acetylation reactions and in hippuric acid synthesis, the two reactions of nicotinuric acid synthesis are catalysed by separate enzymes, though evidence for this can only be obtained by further purification of the system.

Assuming that two enzymes are involved in nicotinuric acid formation, it is uncertain whether they are the same as those synthesizing hippuric acid. The enzyme preparation used in the present experiments was very similar to that employed by Schachter & Taggart (1954) in studies of hippuric acid synthesis, and it was shown chromatographically to be able to form hippuric acid from benzoic acid and glycine. However, it was relatively little purified from the crude extract, and it is quite possible that two or more enzymes catalysing the same type of reaction but having different specificities were present. The same considerations apply to any attempt to identify the enzyme catalysing the activation of nicotinic acid with any of the known enzymes bringing about acyl-CoA formation.

SUMMARY

1. An enzyme preparation has been obtained from acetone-dried ox-liver mitochondria which synthesized nicotinuric acid from nicotinic acid, glycine, adenosine triphosphate and coenzyme A. It also formed nicotinoylhydroxamic acid, which was identified chromatographically, when glycine was replaced by high concentrations of hydroxylamine.

2. Both reactions occurred without added coenzyme A but showed about 50% stimulation in the presence of coenzyme A (40 units/ml.). With a Dowex-1 treated enzyme solution, the syntheses in the absence of added coenzyme A were decreased several-fold, and in both cases a 260–270% increase in activity was found when coenzyme A (40 units/ml.) was added.

3. The mechanisms of these reactions are discussed.

I wish to thank Professor Sir Hans A. Krebs, F.R.S., and Dr W. H. Elliott for their advice and interest in this work. I am indebted to the Christopher Welch Trust, to St John's College, Oxford, and to the Medical Research Council for the

award of Scholarships held during the course of this work. The work was aided by a grant to the Department from the Rockefeller Roundation.

REFERENCES

Beinert, H., Green, D. E., Hele, P., Hift, H., von Korff, R. W. & Ramakrishnan, C. V. (1953). *J. biol. Chem.* **203**, 35.
Chantrenne, H. (1951). *J. biol. Chem.* **189**, 227.
Elliott, W. H. (1956*a*). *Biochem. J.* **62**, 427.
Elliott, W. H. (1956*b*). *Biochem. J.* **62**, 433.
Elliott, W. H. (1957). *Biochem. J.* **65**, 315.
Jones, K. M. (1959). *Biochem. J.* **73**, 719.
Jones, K. M. & Elliott, W. H. (1959). *Biochem. J.* **73**, 706.
Kaplan, N. O. & Lipmann, F. (1948). *J. biol. Chem.* **174**, 37.
Kornberg, A. & Pricer, W. E., jun. (1953). *J. biol. Chem.* **204**, 329.
Lipmann, F., Jones, M. E., Black, S. & Flynn, R. M. (1952). *J. Amer. chem. Soc.* **74**, 2384.
Lynen, F., Reichert, E. & Rueff, L. (1951). *Liebigs Ann.* **574**, 1.
Mahler, H. R., Wakil, S. J. & Bock, R. M. (1953). *J. biol. Chem.* **204**, 453.
Schachter, D. & Taggart, J. V. (1953). *J. biol. Chem.* **203**, 925.
Schachter, D. & Taggart, J. V. (1954). *J. biol. Chem.* **208**, 263.
Stadtman, E. R. & Kornberg, A. (1953). *J. biol. Chem.* **203**, 47.
Stadtman, E. R., Novelli, G. D. & Lipmann, F. (1951). *J. biol. Chem.* **191**, 365.

A Chromatographic Method for the Estimation of Nicotinuric Acid and Nicotinamide

By K. M. JONES
Department of Biochemistry, University of Oxford

(*Received* 14 *April* 1959)

The published methods for the estimation of nicotinuric acid in mixtures containing nicotinic acid and other related compounds depend either upon the colorimetric estimation of 'total nicotinic acid' before and after hydrolysis, different methods of hydrolysis being employed to convert nicotinuric acid and other derivatives into nicotinic acid (for example, Ellinger & Abdel Kader, 1949), or upon the microbiological assay of nicotinic acid with two micro-organisms, one of which also responds to nicotinuric acid whereas the other does not (Johnson, 1945). Since they measure nicotinuric acid by difference, these methods are not able accurately to estimate small amounts of this substance in the presence of a large excess of nicotinic acid, and consequently were unsuitable for use in the experiments described in the two previous papers (Jones & Elliott, 1959; Jones, 1959).

This paper describes a direct method for nicotinuric acid estimation, in which nicotinuric acid is first separated from interfering substances by paper chromatography and then estimated by measurement of its u.v. absorption after elution from the paper. This method has also been successfully adapted to the estimation of nicotinamide in the presence of nicotinic acid.

EXPERIMENTAL

Materials and general methods have been described by Jones & Elliott (1959).

Estimation of nicotinuric acid. Samples containing 5–100 μg. of nicotinuric acid were spotted on to Whatman no. 3 paper to produce spots of 1 cm. diameter. The chromatograms were then developed in descending butan-1-ol saturated with water, for 18–48 hr., the solvent being allowed to drip off the bottom of the papers. After solvent development, chromatograms were dried for 30 min. at 50–60°. The spots of nicotinuric acid were located by making a contact print of the chromatogram by the method of Markham & Smith (1949) with unfiltered light from a mercury lamp. By making a tracing of the photograph and transferring this on to the chromatogram, the positions of the spots could be fixed exactly.

The spots of nicotinuric acid were cut out, each with a border the size of which was then adjusted so that all the pieces were of equal weight. It was generally possible to include the whole of the nicotinuric acid spot in 200 mg. of paper (about 10 cm.^{2}). The pieces of paper were cut into strips 1–2 mm. wide and put in test tubes. A volume (5 ml.) of N-HCl was then added to each tube and they were left to stand with occasional shaking for 3 hr., in which period elution was found to be complete. After 3 hr. the fluid was poured off, centrifuged to remove small shreds of paper and its extinction was measured at 262 mμ against N-HCl. Simultaneously a piece of blank paper was cut from the band having the same R_F as nicotinuric acid and eluted exactly as described above. The extinction of this eluate constituted the 'paper blank' which was deducted from the experimental values. The amount of nicotinuric acid was calculated from a calibration curve separately constructed as described below.

Estimation of nicotinamide. Nicotinamide was estimated by the method described above for nicotinuric acid estimation except that chromatograms were developed for only

12 hr., and the extinctions of the final eluates were read at 261 mμ, which is the wavelength of maximum absorption for nicotinamide in N-HCl. Since nicotinamide had a high R_F in butan-1-ol–water and its running was not greatly affected by the presence of salts, an adequate separation from other u.v.-absorbing substances was obtained with the shorter running time.

Notes on the estimation of nicotinuric acid

Chromatography. The chromatographic separation was markedly influenced by the amount of other substance in the samples. With the incubation mixtures used in the two previous papers (Jones & Elliott, 1959; Jones, 1959) satisfactory separations were obtained in 24–30 hr. with 0·2–0·6 ml. samples. If the incubation mixtures contained appreciable quantities of sucrose, however, chromatography had to be continued for 40–48 hr.

The method of locating the nicotinuric acid spots was found to be entirely satisfactory. If a spot, located as described, was cut from a chromatogram together with a border 1 mm. wide all round and the remainder of the chromatogram was then treated with cyanogen bromide and *p*-aminobenzoic acid, no nicotinuric acid could be detected on the surrounding paper.

Spectrum. The absorption spectrum of synthetic nicotinuric acid was measured and compared with that of nicotinic acid between 210 and 290 mμ, 0·1 mM solutions in water (Fig. 1) and in N-HCl (Fig. 2) being used. Nicotinuric acid has a higher molecular-extinction coefficient than nicotinic acid in both water and N-HCl over the whole of the range recorded, though the position of the maximum at about 262 mμ is virtually the same in each. As with nicotinic acid, the peak in the nicotinuric acid spectrum at 262 mμ is much sharper in N-HCl than in water and the molecular-extinction coefficient at this wavelength is also higher, so that N-HCl was chosen for the elution of nicotinuric acid from the chromatograms.

A sample of synthetic nicotinuric acid which had been chromatographed in butan-1-ol–water and eluted with N-HCl was found, when measured against a paper blank, to have the same absorption maximum as nicotinuric acid in pure solution in N-HCl. Variation in the strength of the HCl in the range from 0·9 to 1·1N led to no change in the extinction given by known amounts of nicotinuric acid or in the position of the absorption maximum.

Paper blank. The practicability of this method depended upon the amount of u.v.-absorbing material eluted from the paper itself ('paper blank'), being small and constant for different parts of a single sheet of paper. To examine these points, blank sheets of Whatman no. 3 paper were chromatographed in butan-1-ol–water for 24 hr. and dried. Several samples of equal weight were cut from the band in which nicotinuric acid would have run, were eluted as described above and the extinctions of the eluates measured at 262 mμ against N-HCl.

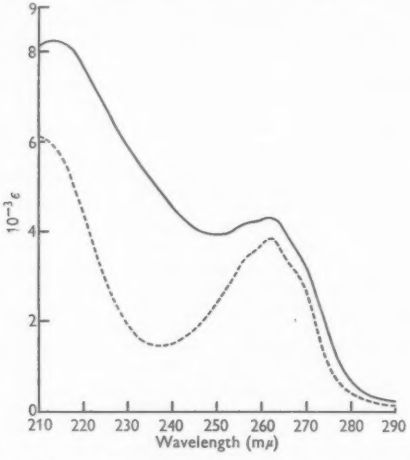


Fig. 1. Spectra of nicotinuric acid and nicotinic acid in water. Readings were taken at 1 mμ intervals (0·5 mμ intervals in the region of the peaks) with 0·1 mM solutions. ——, Nicotinuric acid, $\lambda_{max.}$ 213 and 262 mμ (ε 8240 and 4310 respectively); - - - -, nicotinic acid, $\lambda_{max.}$ 262 mμ (ε 3840).

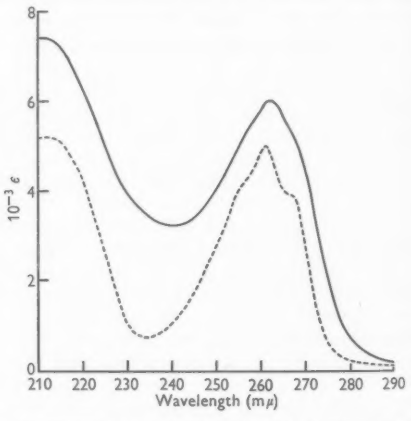


Fig. 2. Spectra of nicotinuric acid and nicotinic acid in N-HCl. Readings were taken at 1 mμ intervals (0·5 mμ intervals in the region of the peaks) with 0·1 mM solutions. ——, Nicotinuric acid, $\lambda_{max.}$ 210·5 and 262 mμ (ε 7440 and 6000 respectively); - - - -, nicotinic acid, $\lambda_{max.}$ 211·5 and 261 mμ (ε 5200 and 5000 respectively).

The extinctions obtained with different samples taken from a single sheet of paper never varied by more than $\pm 0{\cdot}006$, which corresponds to less than $\pm 1\,\mu$g. of nicotinuric acid. Although the paper blank was for practical purposes constant for a single chromatogram, it did vary somewhat from one chromatogram to another, generally falling in the range 0·04–0·08/200 mg. of paper. The main factors determining the magnitude of the paper blank seemed to be the time and the temperature at which the chromatogram was dried. It was not significantly reduced by washing the paper with 2N-acetic acid or 0·5 % disodium ethylenediaminetetra-acetate before chromatography. Since $1\,\mu$g. of nicotinuric acid corresponds to an extinction of about 0·007, it is evident that neither the absolute size nor the variation of the paper blank precludes the possibility of estimating accurately quantities of nicotinuric acid of the order of a few micrograms.

'Zero time' and 'no nicotinic acid' blanks. The possibility of interference with the estimations by the presence in the incubation mixtures of u.v.-absorbing substances other than nicotinuric acid but having similar R_F values was examined in two ways. First, the u.v.-absorbing substances running in the nicotinuric acid area were estimated in an incubation mixture stopped at zero time ('zero time' blank). The mixture had the following composition, which is essentially the same as that used in experiments on nicotinuric acid synthesis (Jones & Elliott, 1959): 0·3 ml. of 0·1M-phosphate buffer, pH 7·35; 0·03 ml. of 0·1M-$MgCl_2$; 0·3 ml. of 0·1M-potassium fumarate; 0·15 ml. of 0·01M-adenosine triphosphate (ATP); 0·2 ml. of 0·308M-glycine; 0·1 ml. of 0·154M-potassium nicotinate; 1·0 ml. of 0·154M-KCl; 1·0 ml. of tissue preparation; final volume, 3·08 ml. It was deproteinized without incubation and a sample chromatographed. The area where nicotinuric acid would have been expected to run and a paper blank were cut out and eluted. The extinction obtained for the 'zero time' blank agreed with the paper blank within the experimental error in several trials.

Secondly, experiments were carried out with mixtures incubated without nicotinic acid ('no nicotinic acid' blank). The mixtures were set up as above, except that they contained 0·154M-KCl in place of potassium nicotinate, and were incubated for 2 hr. at 38°. After incubation, but before deproteinization, appropriate volumes of 0·154M-potassium nicotinate and water were added. Samples were chromatographed and the nicotinuric acid area was eluted. The extinctions obtained for the 'no nicotinic acid' blank agreed with the paper blank over a wide range of nicotinic acid concentrations.

These experiments showed that no u.v.-absorbing material interfering with nicotinuric acid estimation (and incidentally no nicotinuric acid) was present in the incubation mixtures either initially or after incubation without nicotinic acid. It was similarly shown that the components of the incubation mixtures used in experiments with the soluble enzyme preparation (Jones, 1959), especially co-enzyme A and hydroxylamine, did not interfere with the estimation of nicotinuric acid by this method. It was therefore assumed that, apart from the paper blank, all u.v.-absorbing material eluted by the method described was nicotinuric acid. In experimental determinations both a paper blank and either a 'zero time' or a 'no nicotinic acid' blank were determined on each chromatogram. If the extinctions differed by more than 0·01 the chromatogram was discarded.

Calibration curves. With this method, linear calibration curves were obtained over the range 0–200 μg. of nicotinuric acid with synthetic nicotinuric acid, both alone and when mixed with nicotinic acid. In order to obtain a calibration curve under experimental conditions incubation mixtures were set up as described above, except that they contained 0·154M-KCl in place of potassium nicotinate. After incubation for 2 hr. at 38°, but before deproteinization, 0·1 ml. of 0·154M-potassium nicotinate and appropriate amounts of nicotinuric acid and water were added. The mixtures were immediately deproteinized and the nicotinuric acid was estimated as described. In this way a calibration curve was obtained which was

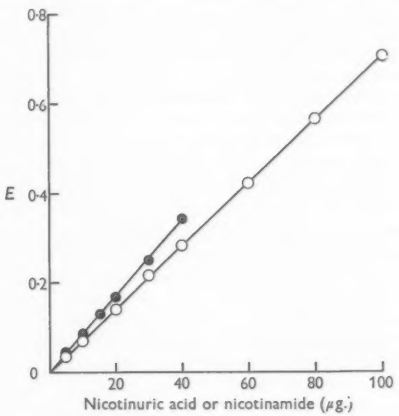


Fig. 3. Calibration of the method of estimation of nicotinuric acid and nicotinamide described in the text. ○, Nicotinuric acid; ●, nicotinamide. The amounts of nicotinuric acid and nicotinamide given refer to the amounts chromatographed.

linear over the range 0–100 μg. of nicotinuric acid on the paper (Fig. 3). Calculation of the slope of the straight line gave a value of 0·00707 for the extinction per μg. of nicotinuric acid (1·273 per μmole). This value was used to calculate the amounts of nicotinuric acid from observed extinctions. A calibration curve or a standard amount of nicotinuric acid was not normally run with each experiment since the agreement between separately determined calibration curves was very good. Occasional determinations of known amounts of nicotinuric acid showed excellent agreement with the expected values.

Notes on the estimation of nicotinamide

Blanks. Measurements of the paper blank in the nicotinamide band showed that the values were similar to those in the nicotinuric acid band and that their variation on a single sheet of paper was small, corresponding to not more than ± 1 μg. of nicotinamide.

Experiments similar to those described above were performed to determine the 'zero time' and 'no nicotinic acid' blanks in the nicotinamide area. It was found that the agreement between these blanks and the paper blank depended upon the tissue preparation used. For example, with rat-kidney mitochondria both the 'zero time' and the 'no nicotinic acid' blanks were the same within the experimental error as the paper blank; with rat-kidney homogenates the 'zero time' blank was the same as the paper blank, but significant amounts of u.v.-absorbing material appeared in incubation mixtures containing no nicotinic acid; and with rat-liver homogenates u.v.-absorbing substances were found in both types of blank. The amounts of u.v.-absorbing substances were in no case very large, corresponding to not more than 10–20 μg. of nicotinamide in a 3 ml. incubation mixture. When samples of the blank incubation mixtures were chromatographed and treated with cyanogen bromide and *p*-aminobenzoic acid it was found that the relative amounts of nicotinamide present in the various mixtures corresponded, as accurately as this could be judged by visual comparison of the spots on the chromatograms, with the amounts found by elution and measurement at 261 mμ. It seemed probable therefore that the u.v.-absorbing material found in the 'zero time' and 'no nicotinic acid' blanks was, in fact, nicotinamide, and for experimental purposes it was assumed that all u.v.-absorbing material found in excess of the paper blank was nicotinamide.

Calibration curves. With nicotinamide alone and with nicotinamide and nicotinic acid linear calibration curves were obtained over the range 0–50 μg. of nicotinamide (higher amounts were not tested). Calibration curves in which nicotinamide was added to the incubation mixtures were also shown to be linear over the range 0–40 μg. of nicotinamide (Fig. 3). The calculated slope of this line gave a value of 0·00854 for the extinction per μg. of nicotinamide (1·042 per μmole).

DISCUSSION

The principal advantage of the method described for nicotinuric acid and nicotinamide estimation is that it is a direct method which is capable of estimating small quantities (0–100 μg.) of these substances in the presence of relatively large amounts of nicotinic acid and other related compounds, with an error of not more than ± 2 μg. These figures apply to the amounts of the substances chromatographed and some restriction is placed upon the sensitivity of the method by the rather small samples which have to be used. The method has proved satisfactory in studies of nicotinuric acid synthesis (Jones & Elliott, 1959; Jones, 1959), and in an unsuccessful search for an enzyme synthesizing nicotinamide from nicotinic acid and ammonia or other amino donors (Jones, 1958).

It must be emphasized that care is necessary in the application of the method. Since it depends upon the separation of nicotinuric acid or nicotinamide from other u.v.-absorbing substances before measurements of extinction are made, it is only specific in so far as the separation is complete. Consequently, in order that the results obtained in any particular case should be valid, it must be shown that such a separation has been achieved.

The chief disadvantage of the method is the long time required to complete an estimation, mainly in the period required for chromatography, but on the other hand it is possible to carry out a number of estimations simultaneously.

SUMMARY

1. A direct method for the estimation of nicotinuric acid and of nicotinamide in the presence of large amounts of nicotinic acid is described. It depends upon the separation of nicotinuric acid or nicotinamide from interfering substances by paper chromatography and the measurement of their u.v. absorption after elution from the paper.

2. Linear calibration curves have been obtained for nicotinuric acid over the range 0–100 μg. and for nicotinamide over the range 0–40 μg.

3. The u.v.-absorption spectrum of nicotinuric acid in water and in N-hydrochloric acid has been measured.

I wish to thank Professor Sir Hans A. Krebs, F.R.S., and Dr W. H. Elliott for their advice and interest in this work. I am indebted to the Christopher Welch Trust, to St John's College, Oxford, and to the Medical Research Council for the award of Scholarships held during the course of this work. The work was aided by a grant to the Department from the Rockefeller Foundation.

REFERENCES

Ellinger, P. & Abdel Kader, M. M. (1949). *Biochem. J.* **44**, 77.
Johnson, B. C. (1945). *J. biol. Chem.* **159**, 227.
Jones, K. M. (1958). D.Phil. Thesis: University of Oxford.
Jones, K. M. (1959). *Biochem. J.* **73**, 714.
Jones, K. M. & Elliott, W. H. (1959). *Biochem. J.* **73**, 706.
Markham, R. & Smith, J. D. (1949). *Biochem. J.* **45**, 294.

Synthesis of ^{35}S-Labelled Arylsulphates by Intact Animals and by Tissue Preparations, with Particular Reference to L-Tyrosine *O*-Sulphate

By A. J. GRIMES
Department of Biochemistry, St Thomas's Hospital Medical School, London, S.E. 1

(*Received* 13 *April* 1959)

The combination of sulphuric acid with phenolic compounds to form arylsulphates is well known to occur in the animal body and certain arylsulphates are present normally in the urine of higher animals. Recently a new arylsulphate, namely L-tyrosine *O*-sulphate, has been reported in the urine of normal and abnormal human subjects (Tallan, Bella, Stein & Moore, 1955). The possible occurrence of this compound in the urine of species other than man has been investigated in the present work.

The synthesis of arylsulphates by tissue preparations has been studied in some detail (e.g. Bernstein & McGilvery, 1952; DeMeio, Wizerkaniuk & Fabiani, 1953; Robbins & Lipmann, 1957; Segal, 1957). Attempts to synthesize L-tyrosine *O*-sulphate, however, from L-tyrosine by various tissue preparations have so far been unsuccessful (Segal, 1957; Dodgson, Rose & Tudball, 1959). Synthesis of this particular arylsulphate has been investigated in the present work with preparations of the liver and kidney tissue from several species. The technique employed was capable of detecting the synthesis of small amounts of arylsulphate and depended on the use of ^{35}S-labelled sulphate as a source of sulphate. The tissue preparations used were active in synthesizing several arylsulphates from phenolic substrates under differing experimental conditions. It was not possible, however, to demonstrate synthesis of L-tyrosine *O*-sulphate from L-tyrosine.

MATERIALS

The potassium salts of phenylsulphuric acid and of *p*-cresylsulphuric acid were prepared by the method of Feigenbaum & Neuberg (1941). The *p*-toluidine salts of the products were prepared (Barton & Young, 1943), and had satisfactory melting points.

L-Tyrosine O-sulphate. The monopotassium salt of this compound was prepared as described by Tallan *et al.* (1955) and was isolated as the dihydrate (Found: N, 4·1; S, 9·4; K, 11·8. Calc. for $C_9H_{10}O_6NSK,2H_2O$: N, 4·2; S, 9·6; K, 11·7%).

Potassium p-ethylphenylsulphate. Freshly distilled chlorosulphonic acid (1·6 ml., 2·9 g.) was added dropwise, and with shaking, to a solution containing 10 ml. of carbon disulphide and 10 ml. of dimethylaniline cooled in a bath containing chipped ice. Freshly distilled *p*-ethylphenol (3 g.) was added to this mixture, which was warmed to room temperature and shaken mechanically for 1 hr. This reaction mixture was added slowly, and with continuous stirring, to an ice-cold solution containing 7 g. of KOH in 10 ml. of water. The resulting mixture was centrifuged at 500 *g* for several minutes and the supernatant liquor was discarded. The precipitate was stirred with 10 ml. of ethanol and the mixture was centrifuged as before. The supernatant was discarded, the precipitate was transferred to a Buchner funnel and was dried by suction. The resulting pale-yellow solid was washed several times with ether and yielded 2·8 g. of material, which was dissolved in 10 ml. of hot water and was rapidly filtered. Large plates were obtained from the pale-yellow filtrate (Found: C, 40·1; H, 3·4; S, 13·3; K, 16·3. Calc. for $C_8H_9O_4SK$: C, 40·0; H, 3·8; S, 13·3; K, 16·3%).

Potassium p-acetylphenylsulphate. This was prepared as described by Dodgson & Spencer (1953) (Found: C, 37·9; H, 2·7; S, 12·4. Calc. for $C_8H_7O_5SK$: C, 37·8; H, 2·8; S, 12·6%).

L-Tyrosine methyl ester. This compound was prepared by the method of Fischer & Schrauth (1907). The product had m.p. 134–136°, $[\alpha]_D^{15}+30°$ in methanol (*c*, 1). Fischer & Schrauth give m.p. 135–136°, $[\alpha]_D^{20}+26°$ in methanol (Found: C, 61·1; H, 6·3; N, 7·2. Calc. for $C_{10}H_{13}O_3N$: C, 61·5; H, 6·7; N, 7·2%).

L-Tyrosine amide. This was prepared from L-tyrosine methyl ester by the method of Blau & Waley (1954). Colourless crystals were obtained, m.p. 152–153°: L-tyrosine amide has m.p. 153–154° (Found: C, 59·8; H, 6·9; N, 15·6. Calc. for $C_9H_{12}O_2N_2$: C, 59·9; H, 6·7; N, 15·5%).

Tyramine O-*sulphate*. This was prepared as described by Dodgson *et al.* (1959). The addition of $Ba(NO_3)_2$ solution to an aqueous solution of the compound produced no precipitate. After the compound had been heated in approx. 2N-HCl solution in a boiling-water bath, the addition of $Ba(NO_3)_2$ solution yielded a precipitate of $BaSO_4$. The compound in aqueous solution gave no colour when diazotized sulphanilic acid solution was added followed by NaOH. After heating the compound in 2N-HCl in a boiling-water bath for several minutes, the addition of a solution of diazotized sulphanilic acid to the cooled solution, followed by NaOH, yielded a red colour. This red was obtained when tyramine solution was treated with diazotized sulphanilic acid followed by NaOH. An aqueous solution of the compound gave a positive ninhydrin test. Paper-chromatographic examination of the compound in three different solvent systems gave evidence of a single spot only. R_F values are shown in Table 2. The compound was identified on chromatograms both by ninhydrin and by diazotized sulphanilic acid. The ultraviolet-absorption spectrum of the compound corresponded to that obtained by Dodgson *et al.* (1959) with tyramine *O*-sulphate.

METHODS

Column chromatography

A column (14·2 cm. × 0·6 cm.) of Dowex 1-X8 (200–400 mesh) in the acetate form was prepared according to Tallan *et al.* (1955); the temperature was held constant by circulating cold tap water through a jacket. Pure L-tyrosine *O*-sulphate placed upon the column was eluted after the passage of 80 ml. of 1·1N-sodium acetate buffer (pH 5·0). The eluate was collected in 2 ml. fractions and the L-tyrosine *O*-sulphate was determined by the photometric ninhydrin method of Stein & Moore (1954). In order to examine urine for the presence of L-tyrosine *O*-sulphate the urine sample was concentrated to half its original volume, the pH was adjusted to 5·0 and the sample was centrifuged. A measured portion (3–5 ml.) of this clear supernatant liquid was applied to the column by pipette.

Paper chromatography

Urine was collected for 24 hr. after the administration of $Na_2{}^{35}SO_4$. The urine specimens were extracted by shaking with an equal volume of butan-1-ol; these extracts were evaporated to dryness *in vacuo* and the residues were dissolved in up to 1 ml. of water. These aqueous solutions, or samples of the original urine, were examined by paper chromatography on Whatman no. 1 paper. The chromatograms were developed overnight by the ascending technique without prior equilibration. The average time of running was 16 hr.

Detection of arylsulphates on chromatograms. (*a*) Colour reagents. To display ninhydrin-sensitive compounds, air-dried chromatograms were dipped in 0·1% (w/v) ninhydrin solution in acetone and again were air-dried. Arylsulphates were detected by suspending air-dried chromatograms in HCl vapour for 10 min. This treatment hydrolyses arylsulphates to H_2SO_4 and the corresponding phenols. The phenols then were displayed by spraying the paper with 0·16% (w/v) diazotized sulphanilic acid solution followed by exposure to NH_3 vapour (Corner & Young, 1955). In the detection of indican on chromatograms, the initial HCl treatment yielded a blue–grey spot which became red on treatment with diazotized sulphanilic acid solution, and darker red after treatment with NH_3. With other arylsulphates no coloured spot was apparent until chromatograms were treated with NH_3 vapour.

(*b*) Radioactivity measurement. Radioactive compounds were detected by passing the strip chromatograms through the automatic recording scanner described by Morrison & Young (1959). Radioactive compounds on the chromatograms gave rise to peaks on an automatic pen-recorded trace.

Where quantitative results were required for experiments with tissue extracts, the areas under the curves obtained on the trace were measured by planimetry and also by cutting out and weighing. It was known by experiment that the radioactivity of compounds on chromatograms was proportional to the areas under the curves obtained on the trace. In order to characterize the arylsulphates synthesized by tissue preparations, R_F values were determined in three different solvent systems. The values so obtained were compared with the R_F values of reference compounds developed at the same time. Results are shown in Table 2.

Housing and dosing of animals

Male rabbits, black-hooded rats, guinea pigs and mice were housed in metabolism cages which permitted the collection of urine separately from faeces. With the exception of rabbits, the animals were maintained on a diet described by Maw (1953). Rabbits were fed on rat cake [J. Murray and Sons (London) Ltd.] and cabbage leaves. The animals had access to water at all times. Each of two rats received 1 mg. of $Na_2{}^{35}SO_4$ (0·5 mc) in 1 ml. of solution, and each of 24 mice received 0·19 mg. of $Na_2{}^{35}SO_4$ (30 μc) in 0·2 ml. of solution by intraperitoneal injection whilst under light ether anaesthesia. A guinea pig received, also by intraperitoneal injection, 1 mg. of $Na_2{}^{35}SO_4$ (0·5 mc) in 1 ml. of solution, and a single rabbit received 10·7 mg. of $Na_2{}^{35}SO_4$ (2·1 mc) in 5 ml. of solution through a stomach tube.

Tissue preparations and incubation

Animals were killed by a blow on the head and the liver or kidney was removed, blotted to remove surface moisture, and was weighed immediately in a chilled container. The tissue was shredded with scissors into an all-glass homogenizer (containing a loose-fitting pestle) cooled to 0°. One of two buffer solutions was used to homogenize the tissue, either 2-amino-2-hydroxymethylpropane-1:3-diol (tris) containing 7·75 ml. of acetic acid/l. of 0·15M-tris, or 0·02M-KH_2PO_4–0·08M-Na_2HPO_4. The pH of these buffer solutions was always in the range 7·27–7·40. The buffer at 0° was added to the tissue so as to yield a 30% (w/v) mixture, which was homogenized. The mixture so obtained was centrifuged at 550 *g* for 7 min. at 0°; the upper layer was removed and is referred to as the 'homogenate'. A sample of this 'homogenate' was centrifuged further at 30 000 *g* for 30 min. at 0°. Three layers were obtained after this centrifuging; the clear layer in the centre was removed and is referred to as the 'supernatant'.

Samples of the 'homogenate' or 'supernatant' fractions (2 ml. unless stated otherwise) were added to stoppered 25 ml. conical flasks containing either 1 or 2 ml. of substrate and supplements dissolved in the buffer (except $MgCl_2$). The composition of a typical reaction mixture, in

which the concentrations of added substances are expressed as molarities in the final mixture, was: adenosine triphosphate (ATP; 7·8 mM) prepared freshly; $MgCl_2,6H_2O$ (7·5 mM) added in water; substrate (0·89 mM); $Na_2{}^{35}SO_4$ (0·68 mM; 60 μc). The final volume was 4 ml. The reaction mixture was shaken while it was incubated in air at 37° for the times indicated. The reaction was stopped by immersing the incubated mixture in a boiling-water bath for 1–2 min. Heating in this way caused no breakdown of the arylsulphates investigated. Coagulated protein was separated by centrifuging, and samples of the clear-yellow supernatant were examined by paper chromatography. Where the incubation resulted in the synthesis of a radioactive arylsulphate, the developed chromatograms contained two radioactive spots. One spot near the origin was due to unchanged free sulphate and the other was due to the arylsulphate synthesized by the tissue preparation. In expressing results quantitatively, the arylsulphate radioactivity has been calculated as a percentage of the total radioactivity. The reproducibility of the method was tested by taking duplicate samples from each of five incubated mixtures in which different amounts of phenylsulphate had been synthesized. Each of these ten samples was examined by paper chromatography on different occasions. Eight of the samples, comprising three duplicate pairs and a sample of each of the other two duplicate pairs, were developed in butan-1-ol–acetic acid–water (4:1:5, by vol.), the remaining two samples were developed in butan-2-ol–aq. 3N-NH_3 soln.–3N-$(NH_4)_2CO_3$ (4:3:3, by vol.). From the developed chromatograms, the amount of phenylsulphate synthesized was calculated. The values obtained indicated agreement between the two samples of each of the five duplicate pairs; they were: 10·7, 10·7; 15·7, 17·1; 24·2, 25·2; 31·3, 32·5 and 36·2, 36·8%. Results quoted in this paper are representative single determinations.

RESULTS

Samples of freshly voided normal human urine were found by column chromatography to contain L-tyrosine *O*-sulphate. When samples of normal male rat-urine concentrates were examined by this technique, no evidence was obtained for the presence of this arylsulphate. L-Tyrosine *O*-sulphate was readily detected when added to normal male rat urine in amount equal to the concentration in normal human urine. The sensitivity of the method used was such that if this arylsulphate had been present in normal male rat urine it was in amount less than 80 μg./24 hr. urine excretion. In view of the inability to detect L-tyrosine *O*-sulphate in normal male rat urine by column chromatography, it was decided to adminster ^{35}S-labelled sulphate to animals and to examine their urine by paper chromatography.

The results are shown in Table 1. After the administration of $Na_2{}^{35}SO_4$ to male rabbits, rats, guinea pigs and mice, it was possible to detect radioactive indican, *p*-cresylsulphate and phenylsulphate in urine concentrates. These three arylsulphates were detected both by their radioactivity and by their colour reactions. No evidence was obtained for the excretion of L-tyrosine *O*-sulphate by any of these species. Butan-1-ol extracts of freshly voided normal human urine were examined also by paper chromatography. It was possible to detect indican, *p*-cresylsulphate and phenylsulphate on the chromatograms.

Studies with tissue preparations

Phenylsulphate-synthesizing activity of various tissues. When attempts to synthesize L-tyrosine *O*-sulphate from L-tyrosine by tissue preparations were unsuccessful, the tissue preparations were tested for arylsulphate-synthesizing activity with phenol as a substrate. The results are shown in Tables 2 and 3. Both 'supernatant' and 'homogenate' fractions of rat-liver preparations synthesized phenylsulphate from phenol in the presence

Table 1. *R_F values of arylsulphates present in butan-1-ol extracts of urine of animals of different species*

With all species, except man, the animals had received $Na_2{}^{35}SO_4$ and the metabolites listed below were radioactive. Those R_F values which are quoted in parentheses are of reference compounds developed simultaneously with the urine samples. Solvent systems (volumes mixed): *A*, butan-1-ol–acetic acid–water (4:1:5); *B*, butan-2-ol–aq. 3N-NH_3 soln.–3N-$(NH_4)_2CO_3$ (4:3:3); *C*, pentan-2-ol–aq. 3N-NH_3 soln.–3N-$(NH_4)_2CO_3$ (4:3:3); *D*, butan-1-ol–pyridine–aq. 3N-NH_3 soln. (4:3:3).

Solvent system	R_F in solvent system: Rat	Guinea pig	Mouse	Rabbit	Man	Chromatograms: HCl vapour	Diazotized sulphanilic acid followed by NH_3 vapour	Metabolite
A	0·45	0·42	—	—	0·45	Blue	Red	Indican
B	0·29	0·28	0·30	0·29	0·26	Blue	Red	Indican
	0·39 (0·41)	0·39 (0·37)	0·42 (0·38)	0·41 (0·37)	0·34 (0·34)	—	Yellow	Phenylsulphate
	0·50 (0·50)	0·44 (0·46)	0·53 (0·47)	0·48 (0·45)	0·40 (0·43)	—	Orange	*p*-Cresylsulphate
C	0·09	0·06	0·07	0·07	0·06	Blue	Red	Indican
	0·16 (0·15)	0·12 (0·11)	0·14 (0·13)	—	0·10 (0·09)	—	Yellow	Phenylsulphate
	0·24 (0·23)	0·20 (0·18)	0·24 (0·21)	0·16 (0·15)	0·18 (0·15)	—	Orange	*p*-Cresylsulphate
D	—	—	0·80	0·75	—	Blue	Red	Indican

Table 2. *R_P values of reference arylsulphates compared with those synthesized by tissue preparations*

Solvent systems were as described in Table 1. The sulphate giving the R_P values in parentheses was not identified.

		R_P in solvent system					
		A		*B*		*D*	
Substrate	Arylsulphate	Reference compound	Metabolite	Reference compound	Metabolite	Reference compound	Metabolite
Phenol	Phenylsulphate	0·49	0·49	0·35	0·34	0·76	0·77
p-Cresol	*p*-Cresylsulphate	0·61	0·61	0·34	0·31	0·77	0·78
p-Ethylphenol	*p*-Ethylphenylsulphate	0·70	0·71	0·62	0·61	0·78	0·79
p-Acetylphenol	*p*-Acetylphenylsulphate	0·54	0·56	0·25	0·25	0·77	0·77
Tyramine	Tyramine *O*-sulphate	0·34	(0·44)	0·10	(0·33)	0·66	(0·35)

Table 3. *Synthesis of phenylsulphate from phenol by 'homogenate' and 'supernatant' fractions prepared from various tissues*

Incubation mixtures were prepared as described in the Methods section. 'Homogenate' and 'supernatant' fractions are called H and S respectively. Incubations were carried out at 37° in air for 1 hr. except where otherwise stated.

		Conversion of sulphate into phenylsulphate (%)	
Expt. no.	Tissue preparation	Phosphate buffer	Tris buffer
1	Rat liver H	2*	—
	S	13*	—
2	H	—	12
	S	—	36
3	S	16	36
4	Rabbit liver H	—	37
	S	—	60
5	H	—	23
	S	—	38
6	Guinea-pig liver H	—	24
	S	—	31
7	Human liver† S	—	34
8	Rat kidney S	—	5
9	Rabbit kidney S	—	5

* Incubated for 2 hr.

† The human liver used was a biopsy sample prepared and incubated within 1·5 hr. of excision.

of added Mg^{2+} ions, ATP and $Na_2{}^{35}SO_4$. The 'supernatant' fractions invariably were more active than the 'homogenate' fractions, irrespective of whether phosphate or tris buffer was used (Table 3, Expts. 1 and 2). When a sample of rat-liver 'supernatant' fraction was prepared and incubated in phosphate buffer, less phenylsulphate synthesis from phenol was observed than when a sample of the same liver was prepared and incubated in tris buffer (Expt. 3). 'Homogenate' or 'supernatant' fractions of the livers of rabbits, guinea pigs and man synthesized phenylsulphate from phenol (Expts. 4, 5, 6 and 7). Rat- and rabbit-kidney 'homogenate' fractions gave no detectable phenylsulphate synthesis from phenol. The 'supernatant' fractions, however, of the kidney tissue of both these species gave small but significant synthesis of phenylsulphate (Expts. 8 and 9).

It is apparent from the results recorded in Table 3 that the tissue preparations examined had arylsulphate-synthesizing activity, as judged by their ability to synthesize phenylsulphate from phenol. Nevertheless, when they were tested for their ability to synthesize L-tyrosine *O*-sulphate from L-tyrosine, negative results were invariably obtained. ATP is required in arylsulphate biosynthesis because the sulphate radical is transferred to the substrate through the intermediary adenosine 3′-phosphate 5′-phosphosulphate (cf. Robbins & Lipmann, 1956). In the absence of ATP in the present experiments, no synthesis of phenylsulphate or L-tyrosine *O*-sulphate occurred when phenol or L-tyrosine was used as substrate with either rat-liver or guinea-pig-liver preparations.

Requirements for metal ions by tissue preparations. The effects of metal ions on arylsulphate biosynthesis has been little investigated. Magnesium, calcium and manganese were examined for their effect on the synthesis of arylsulphates from phenol and from L-tyrosine. The concentrations of the added ions are not necessarily optimum; they are expressed as molarities in the final incubation mixture. Added magnesium (7·5 mM) was not essential for phenylsulphate synthesis from phenol by rat- or guinea-pig-liver preparations in tris buffer. In one experiment with a rat-liver 'supernatant' preparation, omission of Mg^{2+} ions had no effect upon the extent (11%) of phenylsulphate synthesis. In a second experiment, with a similar preparation, 34% conversion of sulphate into phenylsulphate occurred, compared with 67% conversion when Mg^{2+} ion (7·5 mM) was present. When a guinea-pig 'supernatant' fraction was incubated without added Mg^{2+} ion conversion of sulphate into phenylsulphate was 9%, compared with 11% when Mg^{2+} ion (7·5 mM) was present in a control experiment.

When Ca^{2+} ion (5 mM) was added instead of Mg^{2+} ion in a rat-liver preparation, no phenylsulphate synthesis from phenol was observed. In the presence of equimolar amounts of added Ca^{2+} (5 mM) and Mg^{2+} ion (5 mM), phenylsulphate synthesis from phenol was less (4%) than in a rat-liver preparation in which Mg^{2+} ion (5 mM) alone was added (11%). Manganese ions appeared to exert an inhibitory effect since phenylsulphate synthesis by a rat-liver 'supernatant' fraction was less (23%) with added Mn^{2+} ion (7·5 mM) than in the absence of either added Mn^{2+} or Mg^{2+} ion (34%). None of these three ions (Mg^{2+}, Ca^{2+} and Mn^{2+}), whether omitted or added to the incubation media, resulted in synthesis of L-tyrosine *O*-sulphate from L-tyrosine in these tissue preparations.

Biosynthesis of other arylsulphates. In view of this difference in behaviour between phenol and L-tyrosine, the ability of tissue preparations to synthesize arylsulphates from phenolic compounds intermediate in structure between phenol and L-tyrosine was investigated. Both the 'homogenate' and 'supernatant' fraction of rat-liver preparations containing added Mg^{2+} ions, ATP and $Na_2{}^{35}SO_4$ were capable of synthesizing the arylsulphates of *p*-cresol, *p*-ethylphenol and *p*-acetylphenol. The extent of synthesis of the arylsulphates of these three phenols, compared in each case with a control phenylsulphate synthesis, is shown in Table 4 (Expts. 13–15). L-Tyrosine had no effect upon the synthesis of phenylsulphate from phenol by rat-liver preparations (Expt. 16).

When tyramine was used as a substrate, incubation with a rat-liver 'supernatant' fraction resulted in the formation of a small but significant amount of sulphate conjugate. This conjugate had a different R_F from that of synthetic tyramine *O*-sulphate in three different solvent systems (Table 2). When tyramine and phenol were used together as substrates in a rat-liver 'supernatant' fraction containing added Mg^{2+} ions, ATP and $Na_2{}^{35}SO_4$, phenylsulphate synthesis only was observed, to the same extent as when phenol alone was used as substrate (Table 4, Expt. 17). No sulphate conjugation was observed from L-tyrosine amide or L-tyrosine methyl ester in a rat-liver 'supernatant' fraction.

Hydrolysis of L-*tyrosine* O-*sulphate.* The occurrence in the tissue preparations used of an enzyme which hydrolyses L-tyrosine *O*-sulphate would provide an explanation for failure to demonstrate synthesis of this compound by these preparations. The possibility that hydrolysis of this arylsulphate occurred in the rat-liver preparations used, was investigated and the results are shown in Table 5. The preliminary incubation of the preparation with phenol, Mg^{2+} ions and ATP, followed by incubation with $Na_2{}^{35}SO_4$ (Expt. 18), resulted in conversion of $^{35}SO_4{}^{2-}$ ion into phenylsulphate to the extent of 18%. A similar value (19%) was obtained when the preliminary incubation medium contained phenol, Mg^{2+} ion, ATP and L-tyrosine *O*-sulphate, and the mixture was incubated subsequently with $Na_2{}^{35}SO_4$ (Expt. 20). These two

Table 4. *Synthesis of arylsulphates by the 'supernatant' fraction of rat liver*

An incubation mixture, containing phenol as substrate, was used as a control with each of the substrates investigated. Tris was used in all experiments. Unless stated otherwise, the substrate concentration (expressed as molarity in the final incubation mixture) was 0·89 mM.

Expt. no.	Substrate	Conversion of sulphate into arylsulphate (%)
13	Phenol	31
	p-Cresol	25
14	Phenol	46
	p-Ethylphenol	56
15	Phenol	45
	p-Acetylphenol	38
16	Phenol (0·3 mM)	11
	Phenol (0·3 mM) and L-tyrosine (0·3 mM)	12
17	Phenol (0·45 mM)	49
	Phenol (0·45 mM) and tyramine (0·45 mM)	50

Table 5. *Effect of* L-*tyrosine* O-*sulphate on the synthesis of phenylsulphate from phenol by the 'supernatant' fraction of rat liver*

Three reaction mixtures in tris buffer were prepared, containing enzyme (1 ml.), Mg^{2+} ions (6·8 mM), phenol (0·82 mM) and ATP (7·6 mM). Other additions were as shown in column 2. The mixtures were incubated for 30 min. in air at 37°. $Na_2{}^{35}SO_4$ (0·38 mM; 20 μC) was then added to each flask and incubation was continued for a further 45 min. Each sample was then assayed for phenylsulphate. Concentrations are expressed as molarities in the final mixture, whose volume was 2·2 ml.

Expt. no.	Addition to reaction mixture	Total radioactivity on chromatograms (counts/min.)	Radioactive phenylsulphate on chromatograms (counts/min.)	Conversion of sulphate into phenylsulphate (%)
18	None	4324	766	18
19	Na_2SO_4 (0·38 mM)	4262	284	7
20	L-Tyrosine *O*-sulphate (0·36 mM)	4192	795	19

experiments showed that L-tyrosine *O*-sulphate had little effect upon phenylsulphate synthesis. Hydrolysis of L-tyrosine *O*-sulphate during the incubation would have liberated SO_4^{2-} ions, which, by diluting the $Na_2{}^{35}SO_4$, would have reduced the value obtained for apparent conversion of the $^{35}SO_4^{2-}$ ions into phenylsulphate. The extent of this dilution effect is shown in Expt. 19, in which Na_2SO_4 was present during the preliminary incubation. Incubation of this mixture subsequently with $Na_2{}^{35}SO_4$ gave a value for conversion of $^{35}SO_4^{2-}$ ions into phenylsulphate of only 7%.

DISCUSSION

The presence of L-tyrosine *O*-sulphate in normal male human urine and its apparent absence from the urine of normal male rabbits, rats, guinea pigs and mice, indicates a species difference in the urinary occurrence of this compound.

Tissue preparations were made from the livers of rabbits, rats, guinea pigs and man, and from the kidney tissue of rabbits and guinea pigs, which were capable of synthesizing phenylsulphate from phenol. With rat-liver preparations this synthesis occurred under a variety of experimental conditions. In addition to phenylsulphate synthesis, rat-liver preparations were capable of synthesizing the arylsulphates of phenol, *p*-cresol, *p*-ethylphenol and *p*-acetylphenol.

Under none of the experimental conditions described in this work, nor with any of the tissues investigated, was it possible to demonstrate synthesis of L-tyrosine *O*-sulphate from L-tyrosine. It is significant that other workers using tissue preparations have failed to demonstrate this synthesis (Segal, 1957; Dodgson *et al.* 1959). The possibility that L-tyrosine *O*-sulphate is formed and hydrolysed subsequently by arylsulphatases present in the enzyme system seems unlikely in view of the results shown in Table 5. Preparations of the type used in the present work contain Type II arylsulphatases (Baum & Dodgson, 1958). These arylsulphatases have been shown to exhibit relatively low affinity towards L-tyrosine *O*-sulphate (Dodgson, Rose & Tudball, 1957; Dodgson *et al.* 1959).

It seems therefore that, with the conditions used, the enzyme system contained in rat-liver tissue can synthesize the corresponding arylsulphates of simple phenols related in structure to L-tyrosine, but not of L-tyrosine itself. When tyramine (i.e. *p*-hydroxyphenylethylamine) was used as a substrate with rat-liver tissue, synthesis of a sulphate conjugate was demonstrated. Evidence at present available suggests that this conjugate is not tyramine *O*-sulphate and there is the possibility that the conjugate is an *N*-substituted sulphate ester. When L-tyrosine amide and L-tyrosine methyl ester were used as substrates in a rat-liver preparation, no evidence was obtained for synthesis of a sulphate conjugate.

Previous workers have commented upon the unusual behaviour of L-tyrosine *O*-sulphate. Dodgson *et al.* (1959) have pointed out that its presence in bovine fibrinogen is atypical of arylsulphates, which are considered generally to be end products of metabolism. These workers have shown also that the behaviour of this arylsulphate with mammalian arylsulphatase C preparations is unexpected, for it does not undergo hydrolysis. Previous data therefore indicate that the behaviour of L-tyrosine *O*-sulphate is unusual in comparison with other arylsulphates. The present work supports this view.

SUMMARY

1. Examination of normal human urine by column and by paper chromatography confirmed the presence of L-tyrosine *O*-sulphate and revealed the presence of phenylsulphate, *p*-cresylsulphate and indican. Examination of the urine of rabbits, guinea pigs, rats and mice dosed with $Na_2{}^{35}SO_4$ revealed the presence of ^{35}S-labelled phenylsulphate, *p*-cresylsulphate and indican, but not of L-tyrosine *O*-sulphate.

2. By the use of a particle-free system prepared from rat liver and incubated in tris buffer, a brief study has been made of the requirements of the sulphate-esterifying system which forms phenylsulphate from phenol. The arylsulphates of *p*-cresol, *p*-ethylphenol and *p*-acetylphenol were synthesized by this system. Liver- and kidney-tissue fractions of rabbits and guinea pigs synthesized phenylsulphate from phenol, as did a sample of fresh human liver.

3. Tyramine underwent conjugation with sulphate, during incubation with rat-liver-tissue preparation. This biosynthetic sulphate conjugate did not appear to be tyramine *O*-sulphate.

4. No evidence could be obtained for formation of L-tyrosine *O*-sulphate from L-tyrosine with any of the preparations used. This finding supports the results of previous workers.

The author is glad to acknowledge support from the Endowment Fund of St Thomas's Hospital. He is grateful to Professor L. Young for encouragement while this work was carried out. The author also thanks Miss Gillian McLean for technical assistance.

REFERENCES

Barton, A. D. & Young, L. (1943). *J. Amer. chem. Soc.* **65**, 294.

Baum, H. & Dodgson, K. S. (1958). *Nature, Lond.*, **181**, 115.

Bernstein, S. & McGilvery, R. W. (1952). *J. biol. Chem.* **198**, 195.
Blau, K. & Waley, S. G. (1954). *Biochem. J.* **57**, 538.
Corner, E. D. S. & Young, L. (1955). *Biochem. J.* **61**, 132.
DeMeio, R. H., Wizerkaniuk, M. & Fabiani, E. (1953). *J. biol. Chem.* **203**, 257.
Dodgson, K. S., Rose, F. A. & Tudball, N. (1957). *Biochem. J.* **67**, 27P.
Dodgson, K. S., Rose, F. A. & Tudball, N. (1959). *Biochem. J.* **71**, 10.
Dodgson, K. S. & Spencer, B. (1953). *Biochem. J.* **53**, 444.
Feigenbaum, J. & Neuberg, C. A. (1941). *J. Amer. chem. Soc.* **63**, 3529.
Fischer, E. & Schrauth, W. (1907). *Liebigs Ann.* **354**, 21.
Maw, G. A. (1953). *Biochem. J.* **55**, 37.
Morrison, A. R. & Young, L. (1959). *Biochem. J.* **71**, 31P.
Robbins, P. W. & Lipmann, F. (1956). *J. Amer. chem. Soc.* **78**, 6409.
Robbins, P. W. & Lipmann, F. (1957). *J. biol. Chem.* **229**, 837.
Segal, H. L. (1957). *Fed. Proc.* **16**, 245.
Stein, W. H. & Moore, S. (1954). *J. biol. Chem.* **211**, 907.
Tallan, H., Bella, S. T., Stein, W. H. & Moore, S. (1955). *J. biol. Chem.* **217**, 703.

The Effect of 'Glucose Cycloacetoacetate' on the Regeneration of Erythrocytes and Restoration of Haemoglobin Level in Experimental Anaemia in Rats

By M. C. NATH and K. G. PRASANNAN
University Department of Biochemistry, Nagpur, India

(*Received* 22 *December* 1958)

That phenylhydrazine brings about experimental anaemia accompanied by liver damage has been shown by several workers (Damodaran & Vijayaraghavan, 1943; Chanutin, Lentz & Ludewig, 1953; Helbig, 1955; Imanishi, 1956). It was reported by Vijayaraghavan & Dunn (1950) that an experimental anaemia induced in mice by the injection of phenylhydrazine (6–8 mg./100 g. body weight) responded to the parenteral administration of vitamin B_{12} by a regular increase in the erythrocytes. It has also been shown by Popper, Koch-Weser & Szento (1949) and Hove & Hardin (1951) that liver damage caused by phenylhydrazine can be prevented by vitamin B_{12}.

One of the important physiological properties of vitamin B_{12} is its effect on the maintenance of reduced glutathione in liver as reported by Dubnoff (1950), and it has been shown by Nath and associates (Nath & Behki, 1958; Behki, Motlag & Nath, 1958) that 'glucose cycloacetoacetate', a crystalline compound formed by condensing glucose with acetoacetate, when hydrolysed with 2N-hydrochloric acid for 10–15 min. has a similar effect in sparing reduced glutathione in acetoacetate-induced diabetes as well as in scorbutic guinea pigs. Glucose cycloacetoacetate, on such hydrolysis, has been shown by Nath & Bhattathiry (1956*a*, *b*) to give rise to 1:2-dienol glucose. It has also been reported by Nath & Saikia (1958, 1959) that hydrolysed glucose cycloacetoacetate, like vitamin B_{12}, can prevent the rise of the ratio total cholesterol/lipid phosphorus in the blood and tissues in experimental atherosclerosis, induced by diets either with a high content of saturated fat or of cholesterol. Therefore hydrolysed glucose cycloacetoacetate was studied to see if it could also behave like vitamin B_{12} in the regeneration of erythrocytes and restoration of haemoglobin in experimental anaemia induced by phenylhydrazine.

MATERIALS AND METHODS

Male albino rats weighing 90–120 g. were selected and divided into three groups of six or seven each. All were fed on a normal stock diet consisting of wheat flour (75%), casein (10%), yeast powder (5%), Hawk & Oser (1947) salt mixture (5%), ground-nut oil (5%) and cod-liver oil (0·1%). Drinking water was given *ad lib.*

Before making the animals anaemic, the initial haemoglobin level and the erythrocyte count in all the animals were determined from the tail blood. Erythrocytes were counted by the usual method with the help of a Neubauer ruling counting chamber. Haemoglobin was estimated by the method of Wong (1928) with 0·1 ml. of blood, by converting it into Fe^{3+} ions by means of conc. H_2SO_4 and potassium persulphate and by using 3N-KSCN for colour development. The red colour formed was compared against that of the standard iron solution with a green filter in a Lange Universal Colorimeter. The haemoglobin value (in g./100 ml.) was obtained by multiplying the total blood iron (mg.) by the factor 1/3·4.

The experiment was performed for 3 weeks. All the animals were first injected with a single dose of 80 mg. of phenylhydrazine hydrochloride/kg. body wt. The solution of phenylhydrazine for injection was prepared as described by Chanutin *et al.* (1953): a weighed amount of recrystallized phenylhydrazine hydrochloride was dissolved in aq.

0·9% NaCl so that the desired dose/100 g. body wt. was present in 0·25 ml. This solution was adjusted to approx. pH 6·0 by dilute NaOH. The solution was injected into the animals subcutaneously.

Group 1 animals were kept as control. Group 2 animals were daily given an injection of 100 mg./100 g. body wt. of a solution of hydrolysed glucose cyloacetoacetate (GCA) subcutaneously. The hydrolysis was done as follows: to 1 g. of crystalline GCA was added 5 ml. of 2N-HCl and the mixture heated in a boiling-water bath for 20 min. It was then cooled and neutralized with NaOH to pH 7·0 and the whole extracted with three times its volume of ether. The aqueous layer was then collected, ether removed and the pH adjusted to 7·2 by adding a few drops of dil. NaOH soln. by the help of Merck's special indicator paper (pH 6·6-8·0). Group 3 animals were daily given an injection of 10 μg. of vitamin B_{12} (BE-DOUZE; Laboratories Grimault, Paris, France)/100 g. body wt. subcutaneously.

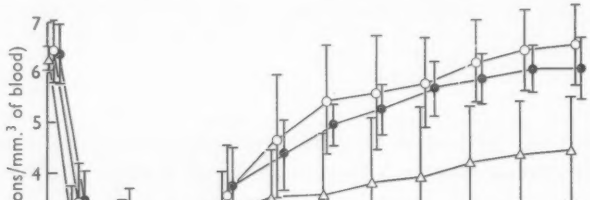


Fig. 1. Effect of daily injections of hydrolysed glucose cycloacetoacetate and of vitamin B_{12} on the regeneration of erythrocytes in experimental anaemic rats. △, Phenylhydrazine hydrochloride (control; six animals); ○, phenylhydrazine hydrochloride and glucose cycloacetoacetate (seven animals); ●, phenylhydrazine hydrochloride and vitamin B_{12} (six animals). The time axes for the three sets of results have been displaced slightly in order to avoid confusion. The vertical lines represent standard deviations from the mean values plotted.

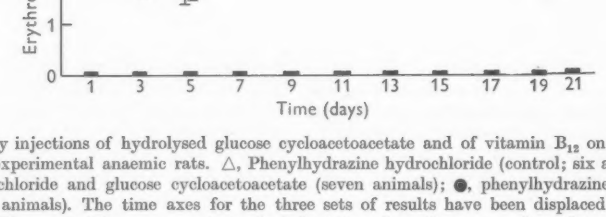


Fig. 2. Effect of daily injections of hydrolysed glucose cycloacetoacetate and of vitamin B_{12} on the haemoglobin levels in experimental anaemic rats. Details are as given for Fig. 1.

The erythrocyte count and haemoglobin estimation were done simultaneously, from the same animal, every alternate day for 21 days. The estimations were made from 8 to 12 noon, before giving them food.

RESULTS AND DISCUSSION

The average values and standard deviations are indicated in Figs. 1 and 2.

After the injection of a single dose of 80 mg. of phenylhydrazine hydrochloride/kg. body wt., a rapid fall in both erythrocyte count and haemoglobin level was noticed within 24 hr. The minimum erythrocyte counts occurred in all three groups at about the fifth day, as observed by Vijayaraghavan & Dunn (1950) and Chanutin *et al.* (1953), and the minimum haemoglobin level was observed at about the same time. Regeneration of erythrocytes and increase in haemoglobin level were observed thereafter in all three groups, the improvement in the experimental groups being slightly but significantly better than that in the control group.

There was a considerable scatter in the individual results in each group, but this was notably least marked in the group receiving vitamin B_{12}.

Some animals receiving 100 mg. of hydrolysed GCA/100 g. body wt. reached a normal erythrocyte count after about 11 days, but none of those receiving 10 μg. of vitamin B_{12}/100 g. body wt. returned to normal before about 15 days. All the control animals showed a slower rate of regeneration of erythrocytes and had cell counts below normal even on the twenty-first day.

No protective effect was found with vitamin B_{12} initially, and the average haemoglobin levels in the group of animals receiving the vitamin were not significantly above those of the control group until the end of the first 2 weeks. Hydrolysed GCA, however, showed a protective effect in a number of cases, and the average haemoglobin levels in the group of animals receiving GCA were slightly but significantly above those in the control group throughout the experiment. There was no significant difference in the effects of hydrolysed GCA and vitamin B_{12} on erythrocyte regeneration or on the haemoglobin level after the first 2 weeks. The experimental animals mostly had normal haemoglobin levels on the twenty-first day but the levels in the control animals remained below normal in spite of the increase noticed day by day. The cell counts appear to be reduced more strongly than the haemoglobin; this is presumably due to the use of the Wong method for estimating haemoglobin, which determines total Fe^{3+} ions.

The beneficial effect of hydrolysed GCA in preference to vitamin B_{12} in the anaemic animals is evident from these results.

Further, it has been shown by Bertolani, Bendandi, Berganini & Chierego (1954) that methionine given to partially hepatectomized rats caused an increase in the rate of hepatic regeneration. Hydrolysed GCA has been found by Nath & Saikia (1959) to help in the biosynthesis of methionine, which might be helpful in the regeneration of hepatic cells damaged by phenylhydrazine.

It was reported by Whipple, Robscheit-Robins & Hooper (1920) and Whipple & Robscheit-Robins (1925) that raw liver, which exerts a beneficial role in pernicious anaemia, contains a blood-forming substance. This is now known to be formed in the stomach from an extrinsic factor (vitamin B_{12}) and Castles' gastric intrinsic factor and is ultimately absorbed through the intestine and stored in the liver. Any substance therefore which can help in the regeneration of the damaged cells of the liver will necessarily help in the restoration of the haematopoietic property.

Further, the recent work of Cohen & Berg (1956) on the formation of haemoglobin from [2-^{14}C]-glycine has indicated that the turnover of labelled haemoglobin is much more in the animals supplied with additional methionine in the diet. The haematopoietic activity of GCA may therefore be accounted for through its effect on the sparing and maintenance of GSH and its help in the biosynthesis of methionine, which might cause an increased rate of hepatic regeneration accompanied by increase in numbers of erythrocytes and turnover of haemoglobin.

SUMMARY

1. The effect of hydrolysed glucose cycloacetoacetate and vitamin B_{12} on the experimental anaemia induced by phenylhydrazine has been studied.

2. Hydrolysed glucose cycloacetoacetate and vitamin B_{12} (100 mg. and 10 μg./100 g. body wt. respectively) have been found to increase the erythrocyte count and haemoglobin level.

3. The mechanism of antianaemic activity of hydrolysed glucose cycloacetoacetate has been discussed.

The authors record their grateful thanks to the Indian Council of Medical Research for a Research Grant.

REFERENCES

Behki, R. M., Motlag, D. B. & Nath, M. C. (1958). *J. sci. industr. Res.* **17C**, 29.

Bertolani, F., Bendandi, A., Berganini, A. & Chierego, G. (1954). *Minerva med., Roma*, **45**, 1160.

Chanutin, A., Lentz, E. A. & Ludewig, S. (1953). *Amer. J. Physiol.* **173**, 474.

Cohen, H. P. & Berg, C. P. (1956). *J. biol. Chem.* **222**, 85.

Damodaran, M. & Vijayaraghavan, P. K. (1943). *Curr. Sci.* **12**, 115.
Dubnoff, J. W. (1950). *Arch. Biochem. Biophys.* **27**, 466.
Hawk, P. B. & Oser, B. L. (1947). *Practical Physiological Chemistry*, 12th ed. p. 1273. London: J. and A. Churchill Ltd.
Helbig, D. (1955). *Z. Kinderheilk.* **77**, 17.
Hove, E. L. & Hardin, J. V. (1951). *Proc. Soc. exp. Biol., N.Y.*, **77**, 502.
Imanishi, K. (1956). *Nagasaki Igakkai Zassi*, **31**, 333.
Nath, M. C. & Behki, R. M. (1958). *Arch. Biochem. Biophys.* **73**, 65.
Nath, M. C. & Bhattathiry, E. P. M. (1956*a*). *Metabolism*, **5**, 11.
Nath, M. C. & Bhattathiry, E. P. M. (1956*b*). *Nature, Lond.*, **178**, 1233.
Nath, M. C. & Saikia, A. (1958). *Indian J. med. Res.* **46**, 579.
Nath, M. C. & Saikia, A. (1959). *Arch. Biochem. Biophys.* **79**, 216.
Popper, H., Koch-Weser, D. & Szento, P. B. (1949). *Proc. Soc. exp. Biol., N.Y.*, **71**, 628.
Vijayaraghavan, P. K. & Dunn, M. S. (1950). *Proc. Soc. exp. Biol., N.Y.*, **75**, 754.
Whipple, G. H. & Robscheit-Robins, F. A. (1925). *Amer. J. Physiol.* **72**, 408.
Whipple, G. H., Robscheit-Robins, F. A. & Hooper, C. W. (1920). *Amer. J. Physiol.* **53**, 236.
Wong, S. Y. (1928). *J. biol. Chem.* **77**, 409.

Formate Metabolism in the Vitamin B_{12}-Deficient Rat

By J. M. NORONHA and A. SREENIVASAN
Department of Chemical Technology, University of Bombay, India

(*Received* 19 *May* 1959)

It was reported earlier (Fatterpaker, Marfatia & Sreenivasan, 1955; Lavate & Sreenivasan, 1956; Kasbekar, Lavate, Rege & Sreenivasan, 1959) that the protective action exerted by vitamin B_{12} in experimentally induced hyperthyroidism in the rat is also observable in terms of a restoration of the uncoupling of oxidative phosphorylation caused by thyroxine. These studies were extended to ascertain whether the incorporation of formate into the purines of liver acid-soluble nucleotides is impaired in the thyrotoxic rat and whether vitamin B_{12} protection is reflected in altered purine synthesis from administered formate.

METHODS

Animals. The basal diet employed was a modification of the corn–soya diet of Ericson, Harper, Williams & Elvehjem (1956) known to cause vitamin B_{12} deficiency, and consisted of (percentages): corn meal 46·2, defatted pea-nut meal 46·2, salts (Ericson *et al.* 1956) 2, L-cystine 0·3, sesame oil 5·0, and vitamin mixture (Ericson *et al.* 1956) 0·3. Rats of either sex (Wistar strain) weighing 70–80 g. were divided into four groups and fed *ad lib.* on the basal diet either as such or with supplements of iodinated casein (Protomone: Cerophyll Laboratories, Kansas City, Mo., U.S.A.) or vitamin B_{12} or both. Iodinated casein known to enhance vitamin B_{12} deficiency (Lewis, Tappan, Register & Elvehjem, 1950) was added at 0·1 g./100 g. and vitamin B_{12} at 20 μg./100 g. Feeding was continued for 7 weeks, during which period the animals on the basal diet with iodinated casein showed some 10% loss in weight when they were considered hyperthyroid (Du Toit, 1952).

Incorporation of formate into adenine and guanine of liver acid-soluble nucleotides. To animals from each group were administered intraperitoneally 0·5 ml. of a solution of 0·1 M-sodium [^{14}C]formate ($2{\cdot}25 \times 10^6$ counts/min.). The animals were operated on under ether anaesthesia 2 hr. later and their livers perfused with cold aq. 0·09% NaCl, excised, weighed and extracted with 40 ml. of ice-cold 6% (w/v) trichloroacetic acid solution for 30 min. The protein precipitate was separated and washed by centrifuging in the cold at 5000 rev./min. The supernatant and washings were combined, neutralized and treated with 5 ml. of 20% (w/v) mercuric acetate solution to precipitate the acid-soluble nucleotides (Kerr, 1942). After separation and washing of the precipitate with 0·5% (w/v) mercuric acetate solution, the nucleotides were freed from Hg by H_2S and the excess of H_2S was removed by aeration. The nucleotides were then hydrolysed in N-H_2SO_4 for 1 hr. over a boiling-water bath. The liberated purines were precipitated at pH 1 with 1 ml. of M-$AgNO_3$ solution (Hayder, Chernigoy, Khatchadourian & Kerr, 1957) and extracted from their silver salts with hot N-HCl. The purine hydrochlorides were freeze-dried and dissolved in 0·2 ml. of N-HCl. The solutions were then chromatographed on Eaton–Dikeman (Mt. Holly Springs, Pa., U.S.A.) no. 613 paper strips, with 65% (v/v) propan-2-ol in 2N-HCl (Hayder *et al.* 1957) as the developing solvent. The separated purines were then identified as their mercury complexes made visible by ammonium sulphide (Vischer & Chargaff, 1948). Segments from the air-dried strips corresponding to the positions of the separated purines were directly eluted in 0·1 N-HCl for adenine and N-HCl for guanine. They were estimated according to the procedure recommended by Vischer & Chargaff (1948) with a Beckman DUV spectrophotometer. Measured portions were evaporated to dryness on 25 mm. diameter Pyrex-glass planchets by heat lamps. Their activities were determined at infinite thinness in a helium-filled SC-16 Flow gas counter with an SC-51 Autoscaler (Tracerlab Inc., Waltham, Mass., U.S.A.) operating at 40–50% efficiency.

Folic acid and vitamin B_{12}. Other animals from each group were killed for determinations in liver of total folic

acid, vitamin B_{12} and formate oxidation. For folic acid assays, 10% (w/v) liver homogenates were prepared in ice-cold water and autolysed overnight in 0·1 M-phosphate buffer, pH 7·2, at 37° under toluene. The samples were then steamed, appropriately diluted, filtered and assayed microbiologically with *Streptococcus faecalis* (Mitbander & Sreenivasan, 1954).

Portions of the liver homogenates were incubated at 37° for 12 hr. under toluene with papain (25 mg./g. of fresh liver) in 0·1 M-acetate buffer, pH 4·6. These samples were then analysed for vitamin B_{12} with *Euglena gracilis* as the test organism according to the method of Hoff-Jørgensen (1954).

Formate oxidation. This was studied at 37° in Warburg vessels. Each flask contained 1·0 ml. of a 20% (w/v) aq. liver homogenate, 1·0 ml. of Krebs–Ringer phosphate buffer, pH 7·2, and 0·066 M-formate in a total volume of 3·0 ml. When used, [^{14}C]formate corresponded to a total activity of $1{\cdot}25 \times 10^4$ counts/min./flask. The centre well contained 0·2 ml. of 10% (w/v) KOH solution. Oxygen uptake was measured for 1 hr. and the CO_2 evolved was trapped in the alkali and later precipitated as $BaCO_3$. The precipitate was washed, plated and counted at infinite thinness in the helium flow-gas counter.

Liver catalase. This was determined by a microcolorimetric method (S. P. Manjrekar, unpublished work). Aq. liver homogenate (1:2000) (2 ml.) was added to 10 ml. of buffered (5·0 mM phosphate buffer, pH 6·8) hydrogen peroxide solution (1·25 mM) at 0°. Portions (2 ml.) of the reaction mixture were pipetted out at different time intervals into 5 ml. of titanium sulphate reagent (Feigl, 1954). The colour developed for residual H_2O_2 was read at 420 mμ in a Klett–Summerson photoelectric colorimeter. Catalase activity was determined in terms of Kat.f. units (Euler & Josephson, 1927), the pseudo-first-order reaction constant K being equal to $D\,(\log x_0 - \log x_t)/t$, where x_0 and x_t are Klett–Summerson readings (corrected for proper blanks) at 0 and t min. and D is the dilution of tissue preparation used for assay.

All results reported are averages for determinations from at least four independent samples ± S.E.M.

RESULTS AND DISCUSSION

The protection by vitamin B_{12} against weight loss caused by iodoprotein feeding, as well as the increased depletion of the vitamin under this condition (Table 1), are in conformity with earlier observations (Fatterpaker *et al.* 1955; Kasbekar *et al.* 1959). There is also a decrease in liver stores of folic acid in experimental thyrotoxicosis. Vitamin B_{12} protection was not, however, observable in terms of a correction of this effect. 'Active formate', as it functions in the exchange and synthesis of C-2 and C-8 of the purine skeleton, is a reduced and formylated derivative of folic acid (Greenberg, Jaenicke & Silverman, 1955). It was therefore of interest to examine whether the decreased levels of folic acid in thyrotoxicosis are reflected in the lowering of [^{14}C]formate incorporation into adenine and guanine of the acid-soluble nucleotides.

Table 1. *Folic acid and vitamin B_{12} levels, and incorporation of [^{14}C]formate into acid-soluble nucleotides in livers of thyrotoxic and vitamin B_{12}-protected rats*

Young adult rats were reared on a vitamin B_{12}-deficient basal diet with or without additions of iodinated casein and vitamin B_{12}. The animals were killed at the end of 7 weeks. The incorporation of formate into the adenine and guanine of liver acid-soluble nucleotides was studied 2 hr. after [^{14}C]formate administration *in vivo*. For details and methods see text.

Diet group	Wt. gain in 7 weeks (g.)	Vitamin B_{12} (μμg./g.)	Folic acid (μg./g.)	Adenine (μμ-moles/g.)	Guanine	[^{14}C]Formate incorporation: Adenine (counts/min./μmole)	Guanine	Adenine (counts/min./g. of liver)	Guanine
Basal	116 ± 5	33 ± 4	3·74 ± 0·05	604 ± 27	43 ± 4	674 ± 10	750 ± 52	428 ± 22	31 ± 4
Thyrotoxic	95 ± 11	24 ± 4	2·96 ± 0·19	725 ± 36	54 ± 7	608 ± 12	660 ± 27	442 ± 27	38 ± 4
Basal + vitamin B_{12}	124 ± 6	162 ± 8	3·70 ± 0·37	721 ± 35	63 ± 8	151 ± 10	139 ± 8	110 ± 12	9 ± 2
Thyrotoxic + vitamin B_{12}	114 ± 5	108 ± 6	2·98 ± 0·15	798 ± 20	66 ± 9	211 ± 14	271 ± 25	166 ± 13	18 ± 3

The observed decrease (Table 1) in specific activity (counts/min./μmole) of liver acid-soluble nucleotide adenine and guanine in the thyrotoxic rat is mostly a dilution effect, explainable in terms of increased endogenous synthesis of these nucleotides in thyrotoxicosis. When the total incorporation/g. of liver is considered, this difference levels off. Guggenheim & Halevy (1957) and Halevy & Guggenheim (1958) have shown that hepatic nucleic acid levels are not affected by various conditions adversely affecting folic acid metabolism, e.g. protein deficiency and incorporation of either succinylsulphathiazole or 4-aminopterin in the diet. Dinning, Cosgrove & Day (1957) have also shown in guinea-pig-liver homogenates that ascorbic acid deficiency depresses the utilization of formate in the synthesis of serine by 78% and in that of methionine by 85%. However, under identical conditions of folic acid metabolism, the incorporation of formate into acid-soluble nucleotides and nucleic acid purines remains remarkably unaffected. It would seem, therefore, that there may be differences in the requirements for 1-C transfer reactions and that, in mild folic acid-deficiency states, systems other than those involved in nucleic acid-purine synthesis are more susceptible.

The incorporation of vitamin B_{12} into the basal or the iodocasein-supplemented rations resulted in decreased incorporation of [^{14}C]formate into acid-soluble nucleotides (Table 1). This decreased incorporation cannot be attributed to dilution of activity due to increased synthesis of acid-soluble nucleotides on vitamin B_{12} administration.

From Table 2 it would seem that oxidation of formate to CO_2 is significantly increased ($P < 0.05$) on vitamin B_{12} administration, partially explaining the observed (Table 1) decrease in formate incorporation into acid-soluble nucleotides. This would suggest a role for vitamin B_{12} in formate metabolism, directly or indirectly linked to formate oxidation. In a recent note Arnstein (1958) has proposed a similar role for vitamin B_{12}.

Table 2. *Oxidation of formate by liver enzymes in thyrotoxic and vitamin B_{12}-protected rats*

For details of grouping, see Table 1 and text. Oxidation of formate by liver enzymes was followed manometrically in conventional Warburg flasks with sodium [^{14}C]formate, the $^{14}CO_2$ being recovered as carbonate in the centre well.

Diet group	O_2 uptake (μl./hr./flask)	Activity of total $^{14}CO_2$ (counts/min.)
Basal	16·0±1·1	8470±318
Thyrotoxic	18·4±2·9	8750±120
Basal + vitamin B_{12}	25·5±4·0	9530±380
Thyrotoxic + vitamin B_{12}	23·6±1·2	9410±320

Since formate oxidation has been linked to catalase activity in rat liver (Nakada & Weinhouse, 1953), it is possible that the decreased formate oxidation in vitamin B_{12} deficiency may be due to an effect of the vitamin on haem-protein synthesis. Catalase activity did not show any appreciable increase in the vitamin B_{12}-supplemented group, the Kat.f. values being 14 220 ± 1730 units for the basal group and 14 940 ± 1460 units for the vitamin B_{12}-supplemented group (C. Bhuvaneswaran, unpublished work).

Other work from this Laboratory would also point to the possibility that the oxidation of rat-liver formate is not entirely linked to catalase activity. It was not enhanced when xanthine was included in the reacting system. Again it was inhibited to a less extent than was catalase by cyanide. For example, 3 mM-cyanide completely inhibits catalase activity, whereas a tenfold concentration of cyanide inhibited formate oxidation only to a negligible extent. Kruhoffer (1951) has shown formate oxidation in rat liver to be diphosphopyridine nucleotide-linked.

Formate oxidation is lowered, but to a less extent in the thyrotoxic rat (Table 2), even though vitamin B_{12} deficiency is augmented. Similarly, the increase in formate oxidation on vitamin B_{12} supplementation is less marked ($0.1 > P > 0.05$) in the iodoprotein-fed group. This would suggest that although administration of iodinated casein intensifies the severity of vitamin B_{12} deficiency it is also accompanied by the development of other unknown metabolic complications. Adverse effects of prolonged iodocasein feeding have been demonstrated in other studies (Fatterpaker *et al.* 1959) with rats and mice, on blood haemoglobin and serum proteins, on the retention of vitamins and soluble sulphydryl compounds in liver, as well as on the ability of liver enzymes to effect conjugation *in vitro* of *p*-aminobenzoic acid and glycine.

SUMMARY

1. An observed decrease in liver folic acid levels in the hyperthyroid rat is not corrected by vitamin B_{12} administration.

2. It is also not accompanied by any clear-cut decrease in the incorporation of [^{14}C]formate *in vivo* into the purines of liver acid-soluble nucleotides.

3. It is concluded that incorporation of formate into the purines of rat liver acid-soluble nucleotides is independent of mild disturbances in folic acid metabolism.

4. Vitamin B_{12} administration to a vitamin B_{12}-deficient rat markedly decreased the incorporation of [^{14}C]formate *in vivo* into liver acid-soluble nucleotides. This could be partly explained in terms of the

accompanying increase in the oxidation of formate studied *in vitro*.

5. Vitamin B_{12} has a role, directly or indirectly, in the oxidation of formate.

6. In rat liver, oxidation of formate may not necessarily be catalase-linked.

We are indebted to the Department of Atomic Energy, Government of India, for a grant from which the expenses of this work were defrayed. Our thanks are due to Mr D. G. Padval for technical assistance and to Mr S. P. Manjrekar for allowing us to give details of his method for determination of catalase activity in advance of its publication.

REFERENCES

Arnstein, H. R. V. (1958). *Biochim. biophys. Acta*, **29**, 652.
Dinning, J. S., Cosgrove, K. W., jun. & Day, P. L. (1957). *J. Nutr.* **61**, 389.
Du Toit, C. H. (1952). In *Phosphorus Metabolism*, vol. 2, p. 597. Ed. by McElroy, W. D. & Glass, B. Baltimore: The Johns Hopkins Press.
Ericson, L. E., Harper, A. E., Williams, J. N., jun. & Elvehjem, C. A. (1956). *J. biol. Chem.* **219**, 59.
Euler, H. von & Josephson, K. (1927). *Liebigs Ann.* **455**, 1.
Fatterpaker, P., Lavate, W. V., Mulgaonkar, A. G., Noronha, J. M., Rege, D. V., Tipnis, H. P. & Sreenivasan, A. (1959). *Brit. J. Nutr.* (in the Press).
Fatterpaker, P., Marfatia, U. & Sreenivasan, A. (1955). *Nature, Lond.*, **176**, 165.
Feigl, F. (1954). In *Spot Tests*, 5th ed., p. 186. Amsterdam: Elsevier Publishing Company.
Greenberg, G. R., Jaenicke, L. & Silverman, M. (1955). *Biochim. biophys. Acta*, **17**, 589.
Guggenheim, K. & Halevy, S. (1957). *J. Nutr.* **63**, 225.
Halevy, S. & Guggenheim, K. (1958). *J. Nutr.* **65**, 77.
Hayder, G. A., Chernigoy, F., Katchadourian, A. & Kerr, S. E. (1957). *J. biol. Chem.* **224**, 707.
Hoff-Jørgensen, E. (1954). In *Methods of Biochemical Analysis*, vol. 1, p. 81. Ed. by Glick, D. New York: Interscience Publishers Inc.
Kasbekar, D. K., Lavate, W. V., Rege, D. V. & Sreenivasan, A. (1959). *Biochem. J.* **72**, 384.
Kerr, S. E. (1942). *J. biol. Chem.* **145**, 647.
Kruhoffer, P. (1951). *Biochem. J.* **48**, 604.
Lavate, W. V. & Sreenivasan, A. (1956). *Nature, Lond.*, **178**, 804.
Lewis, V. J., Tappan, D. V., Register, U. D. & Elvehjem, C. A. (1950). *Proc. Soc. exp. Biol., N.Y.*, **74**, 568.
Mitbander, V. B. & Sreenivasan, A. (1954). *Arch. Mikrobiol.* **21**, 60.
Nakada, H. I. & Weinhouse, S. (1953). *Arch. Biochem. Biophys.* **42**, 257.
Vischer, E. & Chargaff, E. (1948). *J. biol. Chem.* **176**, 703.

Index of Authors

Note. Pages in *Proceedings of the Biochemical Society* are distinguished by P.

ABOU AKKADA, A. R., HOBSON, P. N. & HOWARD, B. H. Carbohydrate fermentation by rumen oligotrich protozoa of the genus *Entodinium* 44P

ACLAND, J. D. The use of a perfusion apparatus to study continuously the transport of ^{131}I-labelled iodide by the small intestine 23P

ADAIR, G. S. *see* PARTRIDGE, S. M. 32P

ADAMS, Y. *see* GERSON, T. 594

ALDRIDGE, W. N. & JOHNSON, M. K. Cholinesterase, succinic dehydrogenase, nucleic acids, esterase and glutathione reductase in subcellular fractions from rat brain 270

ANSELL, G. B. & SPANNER, S. Observations on brain sphingomyelin metabolism 3P

APPLEBY, C. A. & MORTON, R. K. Lactic dehydrogenase and cytochrome b_2 of baker's yeast: enzymic and chemical properties of the crystalline enzyme 539

ARCHIBALD, A. R. & MANNERS, D. J. Studies on carbohydrate-metabolizing enzymes. 2. Trans-α-glucosylation by extracts of *Tetrahymena pyriformis* 292

ARNSTEIN, H. R. V. Changes in the metabolism of DL-[β-^{14}C]serine and [^{14}C]formate in the vitamin B_{12}-deficient rat 23P

ASATOOR, A. M. & DALGLIESH, C. E. Amines in blood and urine 26P

AVI-DOR, Y. *see* GONDA, O. 583

AVIGAD, G. Synthesis of glycosylfructoses by the action of a yeast α-glucosidase 587

BACON, J. S. D. The trisaccharide fraction of some monocotyledons 507

BACON, J. S. D., DEKOCK, P. C. & PALMER, M. J. Aconitase levels in the leaves of iron-deficient mustard plants 41P

BAKER, R. W. R. & PORCELLATI, G. The separation of nitrogen-containing phosphate esters from brain and spinal cord by ion-exchange chromatography 561

BALÁZS, R. *see* LAGNADO, J. R. 18P

BANKS, W. & GREENWOOD, C. T. The starch of the tuber and shoots of the sprouting potato 237

BARTHOLOMEW, M. E., TUPPER, R. & WORMALL, A. Incorporation of ^{65}Zn in the subcellular fractions of the liver and spontaneously occurring mammary tumours of mice after the injection of zinc–glycine containing ^{65}Zn 256

BASSHAM, J. A., BIRT, L. M., HEMS, R. & LOENING, U. E. Determination of the reduced and oxidized pyridine nucleotides in animal tissues 491

BAUCHOP, T. & DAWES, E. A. Pyruvic and formic acid metabolism in *Zymosarcina ventriculi* 17P

BAUDHUIN, P. *see* BEAUFAY, H. 623, 628

BEAUFAY, H., BENDALL, D. S., BAUDHUIN, P. & DE DUVE, C. Tissue fractionation studies. 12. Intracellular distribution of some dehydrogenases, alkaline deoxyribonuclease and iron in rat-liver tissue 623

BEAUFAY, H., BENDALL, D. S., BAUDHUIN, P., WATTIAUX, R. & DE DUVE, C. Tissue fractionation studies. 13. Analysis of mitochondrial fractions from rat liver by density-gradient centrifuging 628

BEAUFAY, H. & DE DUVE, C. Tissue fractionation studies. 9. Enzymic release of bound hydrolases 604

BEAUFAY, H., VAN CAMPENHOUT, E. & DE DUVE, C. Tissue fractionation studies. 11. Influence of various hepatotoxic treatments on the state of some bound enzymes in rat liver 617

BEAUFAY, H. *see also* DE DUVE, C. 610

BEECHEY, R. B. & HOLTON, F. A. Autonomic reversible swelling in heart-muscle mitochondria (sarcosomes) 29P

BELL, M. E. *see* GERSON, T. 594

BENDALL, D. S. *see* BEAUFAY, H. 623, 628

BERGEL, F. & BRAY, R. C. The chemistry of xanthine oxidase. 4. The problems of enzyme inactivation and stabilization 182

BERGER, A. *see* NEUMANN, H. 33

BERRY, J. F. & WHITTAKER, V. P. The acyl-group specificity of choline acetylase 447

BETEL, I. *see* GREENGARD, P. 637

BHARGAVA, P. M., BISHOP, M. W. H. & WORK, T. S. Incorporation of [^{14}C]amino acids into the proteins of bull spermatozoa 247

BHARGAVA, P. M., BISHOP, M. W. H. & WORK, T. S. The chemical composition of bull semen with special reference to nucleic acids, free nucleotides and free amino acids 242

BIRKINSHAW, J. H. Quinones in micro-organisms: chemistry and biosynthesis 1P

BIRT, L. M. *see* BASSHAM, J. A. 491

BISHOP, M. W. H. *see* BHARGAVA, P. M. 242, 247

BLUM, J. J. & LING, N.-S. Oxidation of serotonin and 5-hydroxyindoles during the denaturation of oxyhaemoglobin 530

BOUGHTON, B. & WHEATLEY, V. R. Studies of sebum. 9. Further studies of the composition of the unsaponifiable matter of human-forearm sebum 144

BOWEN, H. J. M. The determination of chlorine, bromine and iodine in biological material by activation analysis 381

BOWMAN, I. B. R., GRANT, P. T., KERMACK, W. O. & OGSTON, D. An inhibitory effect of mepacrine on the catabolism of glucose by *Plasmodium berghei* 42P

BOYD, G. S. & MAWER, E. B. The effect of dietary linoleic acid on serum cholesterol in the rat 9P

BOYD, G. S. *see also* REID, R. H. P. 9P

BOYLAND, E. & SIMS, P. The biochemistry of aromatic amines. 6. The metabolism of 3:4-dimethylaniline in rats 377

BRAY, H. G., FRANKLIN, T. J. & JAMES, S. P. The formation of mercapturic acids. 3. *N*-Acetylation of *S*-substituted cysteines in the rabbit, rat and guinea pig 465

BRAY, R. C. The chemistry of xanthine oxidase. 6. Variations in stability and the presence of an inhibitor in certain preparations 690

BRAY, R. C., MALMSTRÖM, B. G. & VÄNNGÅRD, T. The chemistry of xanthine oxidase. 5. Electron-spin resonance of xanthine oxidase solutions 193

BRAY, R. C. *see also* BERGEL, F. 182

BRITTON, H. G. The detection of carbohydrates with silver in the presence of borate 19P

BROOKES, P., CRATHORN, A. R. & HUNTER, G. D. Site of synthesis of the peptide component of the cell wall of *Bacillus megaterium* 396

BROOKES, P. *see also* HUNTER, G. D. 369

BROOKS, S. A., LAWRENCE, J. C. & RICKETTS, C. R. The phosphate esters of mammalian skin maintained on glucose and various deoxyglucoses 566

BROWN, L. *see* POPJÁK, G. 33P

BROWNHILL, T. J., JONES, A. S. & STACEY, M. The inactivation of ribonuclease during the isolation of ribonucleic acids and ribonucleoproteins from yeast 434

BURMA, D. P. *see* CHAKRAVORTY, M. 48

BUTLER, J. A. V. *see* HUNTER, G. D. 369

CAIN, R. B. *see* CARTWRIGHT, N. J. 305

CALLAGHAN, O. H. & WEBER, G. Kinetic studies on rabbit-muscle myokinase 473

CAMPBELL, P. N. & KERNOT, B. A. Studies on the synthesis of serum albumin by liver microsomes 24P

CARNIE, J. A. & PORTEOUS, J. W. Rabbit intestinal invertase 48P

CARNIE, J. A. & PORTEOUS, J. W. The kinetic properties of invertases obtained from different genera of sheep-rumen holotrich protozoa 47P

CARPENTER, K. J., JONES, W. L. & MASON, E. L. A coloured reaction product from (*im*-DNP)histidine and methoxycarbonyl chloride 11P

CARTWRIGHT, N. J. & CAIN, R. B. Bacterial degradation of the nitrobenzoic acids. 2. Reduction of the nitro group 305

CHADWICK, C. S., NAIRN, R. C. & MCENTEGART, M. G. Investigation of the unreacted fluorescent material in fluorescein–protein conjugates 41P

CHAKRAVORTY, M. & BURMA, D. P. Enzymes of the pentose phosphate pathway in the mung-bean seedling 48

CHARLWOOD, P. A. Ultracentrifugal examination of digestion products from rabbit γ-globulin. *Addendum to:* The hydrolysis of rabbit γ-globulin and antibodies with crystalline papain (Porter, R. R.) 126

CHRISTIE, A. O. & PORTEOUS, J. W. The nutritional requirements of *Actinomyces israeli* 47P

CLIFFE, E. E. & WALEY, S. G. Mechanism of the glyoxylase I reaction and competitive inhibition by ophthalmic acid 25P

COHN, P. The incorporation of amino acids into a ribonucleoprotein fraction of microsomes: a possible pathway of protein synthesis 25P

CONCHIE, J. & HAY, A. J. Mammalian glycosidases. 2. Properties of α-mannosidase and β-galactosidase from rat epididymis 327

COOK, R. P. *see* MOORE, C. H. 43P

COURTS, A. The distribution of mucoprotein in gelatins and fractionated gelatins 600

COURTS, A. The *N*-terminal amino acid residues of gelatin. 4. Chain weight and rigidity relationship in fractionated gelatins 596

CRATHORN, A. R. *see* BROOKES, P. 396; HUNTER, G. D. 369

CRAWHALL, J. C. *see* WATTS, R. W. E. 277

CZERKAWSKI, J. W. *see* HALL, D. A. 356

DAGLEY, S. & STOPHER, D. A. A new mode of fission of the benzene nucleus by bacteria 16P

DALGLIESH, C. E. *see* ASATOOR, A. M. 26P

DAVENPORT, H. E. Coenzyme reduction by illuminated chloroplasts 45P

DAVIES, B. H. & GOODWIN, T. W. Preliminary observations on the incorporation of [2-^{14}C]acetate and [2-^{14}C]mevalonate into the unsaponifiable matter of isolated chromatophores of *Rhodospirillum rubrum* 10P

DAVIS, H. F. *see* PARTRIDGE, S. M. 32P

DAVISON, A. N. & DOBBING, J. Phospholipid metabolism in nervous tissue. 1. A reconsideration of brain and peripheral-nerve phospholipid metabolism *in vivo* 701

DAWES, E. A. *see* BAUCHOP, T. 17P

DAWKINS, M. J. R., JUDAH, J. D. & REES, K. R. The mechanism of action of chlorpromazine. 2. Reduced diphosphopyridine nucleotide–cytochrome *c* reductase and coupled phosphorylation 16

DE DUVE, C. & BEAUFAY, H. Tissue fractionation studies. 10. Influence of ischaemia on the state of some bound enzymes in rat liver 610

DE DUVE, C. *see also* BEAUFAY, H. 604, 617, 623, 628

DE WAARD, A. & POPJÁK, G. Studies on the biosynthesis of cholesterol. 9. Formation of phosphorylated derivatives of mevalonic acid in liver-enzyme preparations 410

DEKOCK, P. C. *see* BACON, J. S. D. 41P

DENNENY, J. M. *see* WINDER, F. G. 500

DILS, R. R. & HÜBSCHER, G. Studies in the incorporation of [*Me*-^{14}C]choline and L-[^{14}C]serine into the phospholipids of subcellular particles of rat liver 26P

DOBBING, J. *see* DAVISON, A. N. 701

DODGSON, K. S. & POWELL, G. M. Studies on sulphatases. 26. Arylsulphatase activity in the digestive juice and digestive gland of *Helix pomatia* 666

DODGSON, K. S. & POWELL, G. M. Studies on sulphatases. 27. The partial purification and properties of the arylsulphatase of the digestive gland of *Helix pomatia* 672

DODGSON, K. S. & TUDBALL, N. Observations on the metabolic fate of the sulphate esters of phenols 27P

DONCK, A. *see* LAURYSSENS, M. 71

DOYLE, J. & SZIRMAI, J. A. The breakdown of hyaluronic acid in rooster comb 28P

DOYLE, J. *see also* SZIRMAI, J. A. 35P

DUNCAN, W. A. M., MANNERS, D. J. & THOMPSON, J. L. Enzyme systems in marine algae. 3. Trans-β-glucosylation by extracts of *Cladophora rupestris* and *Ulva lactuca* 295

DUTTON, G. J. The mechanism of glucuronide formation: a review 29P

EGGLESTON, L. V. & KREBS, H. A. Permeability of *Escherichia coli* to ribose and ribose nucleotides 264

ELLIOTT, W. H. *see* JONES, K. M. 706

ENNOR, A. H. *see* ROSENBERG, H. 521

EVANS, W. C. *see* FERNLEY, H. N. 22P; RIBBONS, D. W. 20P, 21P

FARMER, T. H. Chromatographic studies with pig adrenocorticotrophin 321

FERNLEY, H. N. & EVANS, W. C. Metabolism of 2:4-dichlorophenoxyacetic acid by soil *Pseudomonas*: isolation of α-chloromuconic acid as an intermediate 22P

FEUER, G. The formation of thyroid hormones *in vivo* after the administration of K^{131}I and thyrotropic hormone 349

FEWSTER, J. A. Phosphorylation of shikimic acid by ultrasonic extracts of micro-organisms 14P

FINCH, L. R. & HELE, P. Amino acid activating systems from pig liver 11P

FOTHERBY, K. A method for the estimation of dehydro-*epi*androsterone in urine 339

FRANKLIN, T. J. *see* BRAY, H. G. 465

FRIEND, J. & MAYER, A. M. The destruction of crocin by isolated chloroplasts 18P

GARTON, G. A., LOUGH, A. K. & VIOQUE, E. The effect of sheep rumen contents on triglycerides *in vitro* 46P

GENT, W. L. G. Boundary electrophoresis of brain homogenates cleared by the action of lysolecithin 6P

GERSON, T., SHORLAND, F. B., ADAMS, Y. & BELL, M. E. Further studies on the metabolism of the (+)-*anteiso*-acids, (+)-12-methyltetradecanoic acid and (+)-14-methylhexadecanoic acid, in the rat 594

GIBBONS, R. A. Chemical properties of two mucoids from bovine cervical mucin 209

GIBBONS, R. A. & GLOVER, F. A. The physicochemical properties of two mucoids from bovine cervical mucin 217

GIBSON, Q. H. *see* SMITH, M. H. 101

GILLESPIE, R. *see* WOOD, H. G. 694

GLOVER, F. A. *see* GIBBONS, R. A. 217

GLOVER, J. *see* MERCER, E. I. 5P

GOLDBERG, A. *see* LOCHHEAD, A. C. 39P

GONDA, O., KALUSZYNER, A. & AVI-DOR, Y. Effect of 1:1:1-trichloro-2:2-di-(*p*-chlorophenyl)ethane (DDT) and related compounds on the adenosine triphosphate–phosphate exchange catalysed by a particulate fraction from the mosquito 583

Goodlad, G. A. J. & Munro, H. N. Diet and the action of cortisone on protein metabolism 343
Goodwin, T. W. & Horton, A. A. Threonine and riboflavin biosynthesis in *Eremothecium ashbyii* 13P
Goodwin, T. W. *see also* Davies, B. H. 10P
Goss, W. A. *see* Katz, E. 458
Gould, M. K. & Rawlinson, W. A. Biochemical adaptation as a response to exercise. 1. Effect of swimming on the levels of lactic dehydrogenase, malic dehydrogenase and phosphorylase in muscles of 8-, 11- and 15-week-old rats 41
Gould, M. K. *see also* Rawlinson, W. A. 44
Grant, J. K. *see* Griffiths, K. 5P
Grant, P. T. & Sargent, J. R. L-α-Glycerophosphate oxidase and the terminal respiratory pathway of the bloodstream form of *Trypanosoma rhodesiense* 42P
Grant, P. T. *see also* Bowman, I. B. R. 42P
Greengard, P., Minnaert, K., Slater, E. C. & Betel, I. Yield of oxidative phosphorylation associated with the oxidation of succinate to fumarate 637
Greenwood, C. T. *see* Banks, W. 237
Griffiths, K. & Grant, J. K. Hydroxylation of 11-deoxycorticosterone by adrenal glands of various mammalian species 5P
Grimes, A. J. Synthesis of ^{35}S-labelled arylsulphates by intact animals and by tissue preparations, with particular reference to L-tyrosine *O*-sulphate 723
Gutfreund, H. & Hammond, B. R. Steps in the reactions of chymotrypsin with tyrosine derivatives 526
Gutfreund, H. & Sturtevant, J. M. Steps in the oxidation of xanthine to uric acid catalysed by milk xanthine oxidase 1

Hall, D. A. & Czerkawski, J. W. The purification of the proteolytic component of elastase 356
Hammond, B. R. *see* Gutfreund, H. 526
Hansen, R. G. *see* Wood, H. G. 694
Harada, T. *see* Spencer, B. 34P
Hardenbrook, H. J. *see* Wood, H. G. 694
Harris, H. Turnover of nuclear and cytoplasmic ribonucleic acid in two types of animal cell, with some further observations on the nucleolus 362
Haslewood, G. A. D. & Ogan, A. U. Comparative studies of 'bile salts'. 12. Application to a problem of rodent classification: bile salts of the cutting-grass, *Thryonomys swinderianus* 142
Hay, A. J. *see* Conchie, J. 327
Heald, P. J. Studies on the phosphoproteins of brain: the intracellular localization in brain of a phosphoprotein involved in the metabolic response of cortical slices to electrical stimulation 132
Heath, H. *see* Hoare, D. S. 679
Hele, P. *see* Finch, L. R. 11P
Hems, R. *see* Bassham, J. A. 491
Heyworth, R. *see* Woollen, J. W. 40P
Hoare, D. S. & Heath, H. The biosynthesis of porphyrins from porphobilinogen by *Rhodopseudomonas spheroides*. 2. The partial purification and some properties of porphobilinogen deaminase and uroporphyrinogen decarboxylase 679
Hobson, P. N. *see* Abou Akkada, A. R. 44P
Holton, F. A. *see* Beechey, R. B. 29P
Horton, A. A. *see* Goodwin, T. W. 13P
Hough, L. & Pridham, J. B. The composition of plum gums 550
Howard, B. H. *see* Abou Akkada, A. R. 44P
Hübscher, G. *see* Dils, R. R. 26P
Hunter, G. D., Brookes, P., Crathorn, A. R. & Butler, J. A. V. Intermediate reactions in protein synthesis by the isolated cytoplasmic-membrane fraction of *Bacillus megaterium* 369
Hunter, G. D. *see also* Brookes, P. 396

Iskrić, S. *see* Keglević, D. 53

James, A. T., Lovelock, J. E. & Webb, J. P. W. The lipids of whole blood. 1. Lipid biosynthesis in human blood *in vitro* 106
James, A. T. *see also* Verbeke, R. 24
James, S. P. *see* Bray, H. G. 465
Jellinck, P. H. Action of mushroom tyrosinase on [16-^{14}C]oestrone 30P
Johnson, A. W. & Mauger, A. B. The isolation and structure of actinomycins II and III 535
Johnson, M. K. *see* Aldridge, W. N. 270
Jones, A. S. *see* Brownhill, T. J. 434
Jones, K. M. A chromatographic method for the estimation of nicotinuric acid and nicotinamide 719
Jones, K. M. The mechanism of nicotinuric acid synthesis 714
Jones, K. M. & Elliott, W. H. The synthesis of nicotinuric acid by rat-kidney preparations 706
Jones, W. L. *see* Carpenter, K. J. 11P
Judah, J. D. *see* Dawkins, M. J. R. 16

Kaluszyner, A. *see* Gonda, O. 583
Katchalski, E. *see* Neumann, H. 33
Katz, E. & Goss, W. A. Controlled biosynthesis of actinomycin with sarcosine 458
Kay, D. The inhibition of bacteriophage multiplication by proflavine and its reversal by certain polyamines 149
Kečkeš, S. *see* Keglević, D. 53
Keglević, D., Supek, Z., Kveder, S., Iskrić, S., Kečkeš, S. & Kisić, A. The metabolism of exogenous ^{14}C-labelled 5-hydroxytryptamine in rats 53
Kermack, W. O. *see* Bowman, I. B. R. 42P
Kernot, B. A. *see* Campbell, P. N. 24P
King, H. K. *see* Sutton, C. R. 43P
Kisić, A. *see* Keglević, D. 53
Kogut, M. & Lightbown, J. W. Isolation and purification of a soluble diaphorase-like enzyme from *Bacillus subtilis* 15P
Kogut, M. *see also* Lightbown, J. W. 14P
Korner, A. The effect of hypophysectomy of the rat and of treatment with growth hormone on the incorporation of amino acids into liver proteins in a cell-free system 61
Krahl, M. E. *see* Manchester, K. L. 39P
Krebs, H. A. *see* Eggleston, L. V. 264
Kushner, D. J. The effect of alcohols on enzyme synthesis in *Bacillus cereus* 17P
Kveder, S. *see* Keglević, D. 53

Lagnado, J. R., Balázs, R. & Richter, D. Some properties of adenosine triphosphatase of rat-brain mitochondria 18P
Lauryssens, M., Verbeke, R., Peeters, G. & Donck, A. Incorporation of sodium [1-^{14}C]hexanoate and sodium hydrogen [^{14}C]carbonate into milk constituents by the perfused cow's udder 71
Lauryssens, M. *see also* Verbeke, R. 24
Lawrence, J. C. *see* Brooks, S. A. 566
Lawrie, R. A., Manners, D. J. & Wright, A. α-1:4-Glucosans. 10. Glycogen structure and *rigor mortis* in mammalian muscles 485
Lawson, D. E. M. *see* Pennock, J. F. 4P
Levin, Y. *see* Neumann, H. 33
Levvy, G. A. & McAllan, A. The *N*-acetylation and estimation of hexosamines 127
Lightbown, J. W. & Kogut, M. Soluble enzymes catalysing the oxidation of reduced diphosphopyridine-nucleotide by molecular oxygen and hydrogen peroxide isolated from *Bacillus subtilis* 14P
Lightbown, J. W. *see also* Kogut, M. 15P
Lindsay, D. B. Non-esterified fatty acids in sheep plasma 10P

LING, N.-S. *see* BLUM, J. J. 530
LOCHHEAD, A. C. & GOLDBERG, A. The enzymic formation of haem by human and rat tissues 39P
LOCK, P. A. *see* SLOANE-STANLEY, G. H. 8P
LOENING, U. E. *see* BASSHAM, J. A. 491
LONG, C. & STAPLES, D. A. Chromatographic separation on alumina of the total cerebroside plus sulphatide from rat brain 7P
LONG, C. & STAPLES, D. A. Determination of neuraminic acid in crude brain lipids 385
LOUGH, A. K. *see* GARTON, G. A. 46P
LOVELOCK, J. E. *see* JAMES, A. T. 106
LOWE, A. E. *see* POPJÁK, G. 33P

MCALLAN, A. *see* LEVVY, G. A. 127
MCENTEGART, M. G. *see* CHADWICK, C. S. 41P
MCILWAIN, H. Protein interactions and metabolic response to stimulating agents in isolated cerebral tissues: histones as inhibitors 514
MCILWAIN, H. Protein interactions which condition metabolic response to electrical pulses in mammalian cerebral tissues 30P
MCILWAIN, H. *see also* MARKS, N. 401
MCINDOE, W. M. Lipoprotein of high lipid content from egg yolk 45P
MACLAGAN, N. F. *see* TOMBS, M. P. 167
MALMSTRÖM, B. G. *see* BRAY, R. C. 193
MANCHESTER, K. L. & KRAHL, M. E. Effect of insulin on the incorporation of ^{14}C from ^{14}C-labelled carboxylic acids and bicarbonate into the protein of isolated rat diaphragm 39P
MANNERS, D. J. *see* ARCHIBALD, A. R. 292; DUNCAN, W. A. M. 295; LAWRIE, R. A. 485
MARKS, N. & MCILWAIN, H. Loss of excitability in isolated cerebral tissues, and its restoration by naturally occurring materials 401
MASON, E. L. *see* CARPENTER, K. J. 11P
MAUGER, A. B. *see* JOHNSON, A. W. 535
MAWER, E. B. *see* BOYD, G. S. 9P
MAYER, A. M. *see* FRIEND, J. 18P
MERCER, E. I. & GLOVER, J. The interconversion of cholesterol, 7-dehydrocholesterol and lathosterol in the rat 5P
MINNAERT, K. *see* GREENGARD, P. 637
MOORE, C. H. & COOK, R. P. Human adipose tissue 43P
MOORE, D. *see* POPJÁK, G. 33P
MOORE, T. & SHARMAN, I. M. The cure of the abnormal liability to haemolysis in vitamin E-deficient rats by *NN'*-diphenyl-*p*-phenylenediamine 31P
MOORE, T. & SHARMAN, I. M. Ubiquinone in rats deficient in vitamin A 6P
MORGAN, H. E., RANDLE, P. J. & REGEN, D. M. Regulation of glucose uptake by muscle. 3. The effects of insulin, anoxia, salicylate and 2:4-dinitrophenol on membrane transport and intracellular phosphorylation of glucose in the isolated rat heart 573
MORROW, P. F. W. *see* WEBB, E. C. 7
MORTON, R. A. Ubiquinones 2P
MORTON, R. A. & PHILLIPS, W. E. J. Unsaponifiable constituents of liver, kidney and heart tissues from vitamin E-deficient rats compared with α-tocopherol-supplemented rats 427
MORTON, R. A. & PHILLIPS, W. E. J. Unsaponifiable constituents of rat tissues in relation to vitamin K status 421
MORTON, R. A. & PHILLIPS, W. E. J. Vitamin A deficiency and the ubiquinone and 'SC' of rat liver: the time factor 32P
MORTON, R. A. & PHILLIPS, W. E. J. Vitamin A deficiency and the ubiquinone and substance SC contents of rat liver: the time factor 416
MORTON, R. A. *see also* PENNOCK, J. F. 4P; PHILLIPS, W. E. J. 430
MORTON, R. K. *see* APPLEBY, C. A. 539
MUNRO, H. N. *see* GOODLAD, G. A. J. 343

NAIRN, R. C. *see* CHADWICK, C. S. 41P
NATH, M. C. & PRASANNAN, K. G. The effect of 'glucose cycloacetoacetate' on the regeneration of erythrocytes and restoration of haemoglobin level in experimental anaemia in rats 729
NEEDHAM, D. M. & WILLIAMS, J. M. Some properties of uterus actomyosin and myofilaments 171
NEUMANN, H., LEVIN, Y., BERGER, A. & KATCHALSKI, E. Pepsin-catalysed transpeptidation of the amino-transfer type 33
NORONHA, J. M. & SREENIVASAN, A. Formate metabolism in the vitamin B_{12}-deficient rat 732

OGAN, A. U. *see* HASLEWOOD, G. A. D. 142
OGSTON, D. *see* BOWMAN, I. B. R. 42P

PALMER, M. J. *see* BACON, J. S. D. 41P
PANT, R. Isolation of lombricine and its enzymic phosphorylation 30
PARTRIDGE, S. M., DAVIS, H. F. & ADAIR, G. S. Osmotic and chemical molecular weights of some derivatives from the mucoprotein of cartilage 32P
PARTRIDGE, S. M. *see also* THOMAS, J. 36P
PEETERS, G. *see* LAURYSSENS, M. 71; VERBEKE, R. 24
PENNOCK, J. F., MORTON, R. A. & LAWSON, D. E. M. Ubiquinone and other unsaponifiable constituents of the cod (*Gadus morrhua*) 4P
PERKINS, H. R. The action of lysozyme on the cell walls of Gram-positive micro-organisms 33P
PHILLIPS, W. E. J. & MORTON, R. A. Effects of vitamin A deficiency and adrenalectomy on ubiquinone and substance SC in rat liver 430
PHILLIPS, W. E. J. *see also* MORTON, R. A. 416, 421, 427, 32P
PLASKETT, L. G. The localization of ^{131}I in biosynthetically labelled [^{131}I]thyroxine 115
POPJÁK, G., LOWE, A. E., MOORE, D., BROWN, L. & SMITH, F. A. Gas–liquid radiochromatography 33P
POPJÁK, G. *see also* DE WAARD, A. 410
PORCELLATI, G. *see* BAKER, R. W. R. 561
PORTEOUS, J. W. *see* CARNIE, J. A. 47P, 48P; CHRISTIE, A. O. 47P
PORTER, R. R. The hydrolysis of rabbit γ-globulin and antibodies with crystalline papain 119
PORTER, R. R. *see also* RYLE, A. P. 75
POWELL, G. M. *see* DODGSON, K. S. 666, 672
PRASANNAN, K. G. *see* NATH, M. C. 729
PRIDHAM, J. B. *see* HOUGH, L. 550
PUMPHREY, A. M. *see* REDFEARN, E. R. 3P

RANDLE, P. J. *see* MORGAN, H. E. 573; TAYLOR, K. W. 12P
RAWLINSON, W. A. & GOULD, M. K. Biochemical adaptation as a response to exercise. 2. Adenosine triphosphatase and creatine phosphokinase activity in muscles of exercised rats 44
RAWLINSON, W. A. *see also* GOULD, M. K. 41
REDFEARN, E. R. & PUMPHREY, A. M. Quinones in electron transport and oxidative phosphorylation 3P
REES, K. R. *see* DAWKINS, M. J. R. 16
REGEN, D. M. *see* MORGAN, H. E. 573
REID, R. H. P. & BOYD, G. S. A method for the specific differential estimation of cholic and chenodeoxycholic acids in human plasma 9P
RIBBONS, D. W. & EVANS, W. C. The metabolic pathway of phthalic acid by soil *Pseudomonas* 20P
RIBBONS, D. W. & EVANS, W. C. The pathway of metabolism of protocatechuic acid by *Pseudomonas* species 21P

RICHMOND, M. H. Incorporation of canavanine by *Staphylococcus aureus* 524 SC 261
RICHMOND, M. H. The differential effect of arginine and canavanine on growth and enzyme formation in *Staphylococcus aureus* 524 SC 155
RICHTER, D. *see* LAGNADO, J. R. 18P
RICKETTS, C. R. *see* BROOKS, S. A. 566
ROSENBERG, H. & ENNOR, A. H. The isolation of lombricine and its possible biological precursor 521
ROWE, C. E. The biosynthesis of phospholipids by human blood cells 438
RYLE, A. P. & PORTER, R. R. Parapepsins: two proteolytic enzymes associated with porcine pepsin 75

SANDERS, F. & SEAMAN, G. R. Purification of the vitamin B_{12}-active material from the haemoflagellate, *Crithidia fasciculata* 580
SARGENT, J. R. *see* GRANT, P. T. 42P
SEAMAN, G. R. *see* SANDERS, F. 580
SHARMAN, I. M. *see* MOORE, T. 6P, 31P
SHORLAND, F. B. *see* GERSON, T. 594
SIMS, P. Metabolism of polycyclic compounds. 14. The conversion of naphthalene into compounds related to *trans*-1:2-dihydro-1:2-dihydroxynaphthalene by rabbits 389
SIMS, P. *see also* BOYLAND, E. 377
SLATER, E. C. *see* GREENGARD, P. 637
SLATER, T. F. Studies on the succinate–neotetrazolium reductase system: activation by vitamin K_3 314
SLOANE-STANLEY, G. H. Aspects of spectrophotometric iodometry 8P
SLOANE-STANLEY, G. H. & LOCK, P. A. Effects of pH and temperature on the reduction of periodates by inositol 8P
SMITH, E. B. The phospholipids in the human aorta 34P
SMITH, F. A. *see* POPJÁK, G. 33P
SMITH, M. H. Kinetics and equilibria in systems containing haem, carbon monoxide and pyridine 90
SMITH, M. H. & GIBSON, Q. H. The preparation and some properties of myoglobin containing meso- and deuterohaem 101
SOUTER, F. *see* TOMBS, M. P. 167
SPANNER, S. *see* ANSELL, G. B. 3P
SPENCER, B. Studies on sulphatases. 25. The determination of $BaS^{16}O_3{}^{18}O$ by infrared spectroscopy 442
SPENCER, B. The ester sulphate pattern of rat liver 19P
SPENCER, B. & HARADA, T. The role of choline sulphate in the sulphur metabolism of fungi 34P
SREENIVASAN, A. *see* NORONHA, J. M. 732
STACEY, M. *see* BROWNHILL, T. J. 434
STAPLES, D. A. *see* LONG, C. 385, 7P
STEVEN, F. S. & TRISTRAM, G. R. The denaturation of ovalbumin: changes in optical rotation, extinction and viscosity during serial denaturation in solutions of urea 86
STICKLAND, R. G. Some properties of oxaloacetate-synthesizing enzyme 660
STICKLAND, R. G. Some properties of the malic enzyme of pigeon liver. 1. Conversion of malate into pyruvate 646
STICKLAND, R. G. Some properties of the malic enzyme of pigeon liver. 2. Synthesis of malate from pyruvate 654
STITCH, S. R. Liquid scintillation-counting for [^{14}C]-steroids 287
STOPHER, D. A. *see* DAGLEY, S. 16P
STURTEVANT, J. M. *see* GUTFREUND, H. 1
SUPEK, Z. *see* KEGLEVIĆ, D. 53
SUTTON, C. R. & KING, H. K. Action of inhibitors on an amino acid decarboxylase: nature of the enzyme centre 43P
SZIRMAI, J. A. & DOYLE, J. The distribution of polysaccharides in hyaline cartilage 35P
SZIRMAI, J. A. *see also* DOYLE, J. 28P

TATA, J. R. The purification and properties of thyroxine deiodinase from skeletal muscle 36P
TAYLOR, K. W., VARGAS, L. & RANDLE, P. J. Some further observations on insulin and insulin antagonists in protein fractions of normal human serum 12P
THOMAS, J. & PARTRIDGE, S. M. The specificity of elastases 36P
THOMPSON, J. L. *see* DUNCAN, W. A. M. 295
THOMSON, R. H. Introduction: distribution and nature of the naturally occurring quinones 1P
TOMBS, M. P., SOUTER, F. & MACLAGAN, N. F. The spectrophotometric determination of protein at 210 mμ 167
TRIM, A. R. Metal ions as precipitants for nucleic acids and their use in the isolation of polynucleotides from leaves 298
TRISTRAM, G. R. *see* STEVEN, F. S. 86
TUDBALL, N. *see* DODGSON, K. S. 27P
TUPPER, R. *see* BARTHOLOMEW, M. E. 256

VAN CAMPENHOUT, E. *see* BEAUFAY, H. 617
VAN HEYNINGEN, R. Metabolism of xylose by the lens. 2. Rat lens *in vivo* and *in vitro* 197
VÄNNGÅRD, T. *see* BRAY, R. C. 193
VARGAS, L. *see* TAYLOR, K. W. 12P
VERBEKE, R., LAURYSSENS, M., PEETERS, G. & JAMES, A. T. Incorporation of DL-[1-^{14}C]leucine and [1-^{14}C]*iso*valeric acid into milk constituents by the perfused cow's udder 24
VERBEKE, R. *see also* LAURYSSENS, M. 71
VIOQUE, E. *see* GARTON, G. A. 46P

WALEY, S. G. *see* CLIFFE, E. E. 25P
WALKER, G. J. & WHELAN, W. J. Structure of glycogen and amylopectin muscle-phosphorylase limit dextrins 20P
WALKER, P. G. *see* WOOLLEN, J. W. 40P
WATTIAUX, R. *see* BEAUFAY, H. 628
WATTS, R. W. E. & CRAWHALL, J. C. The first glycine metabolic pool in man 277
WEBB, E. C. & MORROW, P. F. W. The activation of an arylsulphatase from ox liver by chloride and other anions 7
WEBB, J. P. W. *see* JAMES, A. T. 106
WEBER, G. *see* CALLAGHAN, O. H. 473
WHEATLEY, V. R. *see* BOUGHTON, B. 144
WHELAN, W. J. *see* WALKER, G. J. 20P
WHITTAKER, V. P. The subcellular localization of transmitter substances in the central nervous system 37P
WHITTAKER, V. P. *see also* BERRY, J. F. 447
WILKINSON, J. H. The biological action of substances related to thyroxine. 11. Acetic acid analogues of thyroxine and tri-iodothyronine 334
WILLIAMS, J. M. *see* NEEDHAM, D. M. 171
WILT, F. H. Thyroxine and visual pigment conversion in larvae of *Rana catesbeiana* 13P
WINDER, F. G. & DENNENY, J. M. Metal-catalysed autoxidation of isoniazid 500
WOOD, H. G., GILLESPIE, R., HANSEN, R. G., WOOD, W. A. & HARDENBROOK, H. J. Arteriovenous $^{14}CO_2$ differences and the pentose cycle in the cow's udder 694
WOOD, W. A. *see* WOOD, H. G. 694
WOODIN, A. M. Fractionation of a leucocidin from *Staphylococcus aureus* 225
WOOLLEN, J. W., HEYWORTH, R. & WALKER, P. G. The specificity of preparations with *N*-acetyl-β-glucosaminidase and *N*-acetyl-β-galactosaminidase activities 40P
WORK, T. S. *see* BHARGAVA, P. M. 242, 247
WORMALL, A. *see* BARTHOLOMEW, M. E. 256
WRIGHT, A. *see* LAWRIE, R. A. 485

Index of Subjects

Note. Pages in *Proceedings of the Biochemical Society* are distinguished by P

Acetate, incorporation of, into blood-cell phospholipids (Rowe, C. E.) 438

[2-^{14}C]Acetate, incorporation of, into unsaponifiable matter of isolated chromatophores of *Rhodospirillum rubrum* (Davies, B. H. & Goodwin, T. W.) 10P

Acetyl-coenzyme A synthetase in brain and liver (Berry, J. F. & Whittaker, V. P.) 447

N-Acetyl-β-galactosaminidase activities, specificity of preparations with *N*-acetyl-β-glucosaminidase and (Woollen, J. W., Heyworth, R. & Walker, P. G.) 40P

N-Acetylglucosamine, preparation of (Levvy, G. A. & McAllan, A.) 127

N-Acetyl-β-glucosaminidase activities, specificity of preparations with *N*-acetyl-β-galactosaminidase and (Woollen, J. W., Heyworth, R. & Walker, P. G.) 40P

Aconitase levels in leaves of iron-deficient mustard plants (Bacon, J. S. D., DeKock, P. C. & Palmer, M. J.) 41P

Actinomyces israeli, nutritional requirements of (Christie, A. O. & Porteous, J. W.) 47P

Actinomycin, controlled biosynthesis of, with sarcosine (Katz, E. & Goss, W. A.) 458

Actinomycins II and III, isolation and structure of (Johnson, A. W. & Mauger, A. B.) 535

Actomyosin, uterus, properties of (Needham, D. M. & Williams, J. M.) 171

Acyl-coenzyme A synthetase in brain and liver (Berry, J. F. & Whittaker, V. P.) 447

Adenosine phosphate, liver, effect of diet and cortisone on (Goodlad, G. A. J. & Munro, H. N.) 343

Adenosine triphosphatase and creatine phosphokinase activity in muscles of exercised rats (Rawlinson, W. A. & Gould, M. K.) 44

Adenosine triphosphatase, properties of, of rat-brain mitochondria (Lagnado, J. R., Balázs, R. & Richter, D.) 18P

Adenosine triphosphate–phosphate exchange, effect of 1:1:1-trichloro-2:2-di-(*p*-chlorophenyl)ethane and related compounds on, catalysed by a particulate fraction from the mosquito (Gonda, O., Kaluszyner, A. & Avi-Dor, Y.) 583

Adipose tissue, human (Moore, C. H. & Cook, R. P.) 43P

Adrenal glands of various mammals, hydroxylation of 11-deoxycorticosterone by (Griffiths, K. & Grant, J. K.) 5P

Adrenocorticotrophin, pig, chromatographic studies with (Farmer, T. H.) 321

Albumin, serum, synthesis of, by liver microsomes (Campbell, P. N. & Kernot, B. A.) 24P

Alcohols, effect of, on enzyme synthesis in *Bacillus cereus* (Kushner, D. J.) 17P

Alcohols in human-forearm sebum (Boughton, B. & Wheatley, V. R.) 144

Amines, content of, in blood and urine (Asatoor, A. M. & Dalgliesh, C. E.) 26P

Amino acid decarboxylase, action of inhibitors on (Sutton, C. R. & King, H. K.) 43P

Amino acid-activating systems from pig liver (Finch, L. R. & Hele, P.) 11P

Amino acids, effect of hypophysectomy of the rat and of treatment with growth hormone on incorporation of, into liver proteins in a cell-free system (Korner, A.) 61

Amino acids, free, of bull semen (Bhargava, P. M., Bishop, M. W. H. & Work, T. S.) 242

Amino acids, incorporation of, into a ribonucleoprotein fraction of microsomes (Cohn, P.) 25P

[^{14}C]Amino acids, incorporation of, into proteins of bull spermatozoa (Bhargava, P. M., Bishop, M. W. H. & Work, T. S.) 247

Amylopectin, structure of muscle-phosphorylase limit dextrins of (Walker, G. J. & Whelan, W. J.) 20P

Anaemia, experimental, effect of 'glucose cycloacetoacetate' on the regeneration of erythrocytes and restoration of haemoglobin level in, in rats (Nath, M. C. & Prasannan, K. G.) 729

Anions, various, activation of an arylsulphatase from ox liver by (Webb, E. C. & Morrow, P. F. W.) 7

Anoxia, effect of, on membrane transport and intracellular phosphorylation of glucose in isolated rat heart (Morgan, H. E., Randle, P. J. & Regen, D. M.) 573

Antibodies, hydrolysis of rabbit γ-globulin and, with crystalline papain (Porter, R. R.) 119

Aorta, human, phospholipids in (Smith, E. B.) 34P

Arginine, differential effect of canavanine and, on growth and enzyme formation in *Staphylococcus aureus* (Richmond, M. H.) 155

Arylsulphatase activity in the digestive juice and digestive gland of *Helix pomatia* (Dodgson, K. S. & Powell, G. M.) 666

Arylsulphatase from ox liver, activation of, by chloride and other anions (Webb, E. C. & Morrow, P. F. W.) 7

Arylsulphatase of the digestive gland of *Helix pomatia*, partial purification and properties of (Dodgson, K. S. & Powell, G. M.) 672

Arylsulphates, ^{35}S-labelled, synthesis of, by intact animals and tissue preparations (Grimes, A. J.) 723

Bacillus cereus, effect of alcohols on enzyme synthesis in (Kushner, D. J.) 17P

Bacillus megaterium, intermediate reactions in protein synthesis by isolated cytoplasmic-membrane fraction of (Hunter, G. D., Brookes, P., Crathorn, A. R. & Butler, J. A. V.) 369

Bacillus megaterium, site of synthesis of peptide component of cell wall of (Brookes, P., Crathorn, A. R. & Hunter, G. D.) 396

Bacillus subtilis, isolation and purification of a soluble diaphorase-like enzyme from (Kogut, M. & Lightbown, J. W.) 15P

Bacillus subtilis, soluble enzymes catalysing oxidation of reduced diphosphopyridine nucleotide by molecular oxygen and hydrogen peroxide isolated from (Lightbown, J. W. & Kogut, M.) 14P

Bacteriophage multiplication, inhibition of, by proflavine and reversal by certain polyamines (Kay, D.) 149

Barium sulphate ($BaS^{16}O_3{}^{18}O$), determination of, by infrared spectroscopy (Spencer, B.) 442

Bentonite, removal of yeast ribonuclease by adsorption on (Brownhill, T. J., Jones, A. S. & Stacey, M.) 434

Benzene nucleus, fission of, by bacteria (Dagley, S. & Stopher, D. A.) 16P

Bicarbonate, effect of insulin on incorporation of ^{14}C from ^{14}C-labelled carboxylic acids and, into protein of isolated rat diaphragm (Manchester, K. L. & Krahl, M. E.) 39P

[^{14}C]Bicarbonate, incorporation of, into milk constituents by perfused cow's udder (Lauryssens, M., Verbeke, R., Peeters, G. & Donck, A.) 71

Bile salts of the cutting-grass (*Thryonomys swinderianus*) (Haslewood, G. A. D. & Ogan, A. U.) 142

Blood cells, human, biosynthesis of phospholipids by (Rowe, C. E.) 438
Blood, content of amines in (Asatoor, A. M. & Dalgliesh, C. E.) 26P
Blood, determination of halogens in (Bowen, H. J. M.) 381
Blood, human, lipid biosynthesis in, *in vitro* (James, A. T., Lovelock, J. E. & Webb, J. P. W.) 106
Borate, detection of carbohydrates with silver in the presence of (Britton, H. G.) 19P
Brain homogenates, boundary electrophoresis of, cleared by the action of lysolecithin (Gent, W. L. G.) 6P
Brain, intracellular localization of phosphoprotein involved in metabolic response to electrical stimulation by (Heald, P. J.) 132
Brain lipids, crude, determination of neuraminic acid in (Long, C. & Staples, D. A.) 385
Brain, phospholipid metabolism of, *in vivo* (Davison, A. N. & Dobbing, J.) 701
Brain, rat, cholinesterase, succinic dehydrogenase, nucleic acids, esterase and glutathione reductase in subcellular fractions from (Aldridge, W. N. & Johnson, M. K.) 270
Brain, rat, chromatographic separation on alumina of total cerebroside plus sulphatide from (Long, C. & Staples, D. A.) 7P
Brain, separation of nitrogen-containing phosphate esters from spinal cord and, by ion-exchange chromatography (Baker, R. W. R. & Porcellati, G.) 561
Brain sphingomyelin metabolism, observations on (Ansell, G. B. & Spanner, S.) 3P
Bromine, determination of, in biological material by activation analysis (Bowen, H. J. M.) 381

Canavanine, differential effect of arginine and, on growth and enzyme formation of *Staphylococcus aureus* (Richmond, M. H.) 155
Canavanine, incorporation of, by *Staphylococcus aureus* (Richmond, M. H.) 261
Carbohydrate fermentation by *Entodinium* (Abou Akkada, A. R., Hobson, P. N. & Howard, B. H.) 44P
Carbohydrates, detection of, with silver in the presence of borate (Britton, H. G.) 19P
Carbohydrates in connective tissues (Courts, A.) 596
Carbon dioxide, radioactive, arteriovenous differences in, and the pentose cycle in cow's udder (Wood, H. G., Gillespie, R., Hansen, R. G., Wood, W. A. & Hardenbrook, H. J.) 694
Carbon monoxide, kinetics and equilibria in systems containing haem, pyridine and (Smith, M. H.) 90
[^{14}C]Carboxylic acids, effect of insulin on incorporation of ^{14}C from bicarbonate and, into protein of isolated rat diaphragm (Manchester, K. L. & Krahl, M. E.) 39P
Carboxymethylcellulose, chromatography of adrenocorticotrophin on (Farmer, T. H.) 321
Cartilage, hyaline, distribution of polysaccharides in (Szirmai, J. A. & Doyle, J.) 35P
Cartilage, osmotic and chemical molecular weights of derivatives from mucoprotein of (Partridge, S. M., Davis, H. F. & Adair, G. S.) 32P
Cell wall of *Bacillus megaterium*, site of synthesis of peptide component of (Brookes, P., Crathorn, A. R. & Hunter, G. D.) 396
Cerebral cortex, intracellular localization of phosphoprotein involved in metabolic response to electrical stimulation by (Heald, P. J.) 132
Cerebral tissues, isolated, histones as inhibitors of protein interactions and metabolic response to stimulating agents in (McIlwain, H.) 514
Cerebral tissues, isolated, loss of excitability in, and restoration by naturally occurring materials (Marks, N. & McIlwain, H.) 401
Cerebral tissues, mammalian, protein interactions which condition metabolic response to electrical pulses in (McIlwain, H.) 30P
Cerebroside, plus sulphatide, total, rat-brain, chromatographic separation of, on alumina (Long, C. & Staples, D. A.) 7P
Chain weight and rigidity relationship in fractionated gelatins (Courts, A.) 596
Chenodeoxycholic acid, specific differential estimation of cholic acid and, in human plasma (Reid, R. H. P. & Boyd, G. S.) 9P
Chloride ion, activation of an arylsulphatase from ox liver by (Webb, E. C. & Morrow, P. F. W.) 7
Chlorine, determination of, in biological material by activation analysis (Bowen, H. J. M.) 381
α-Chloromuconic acid, isolation of, as intermediate in metabolism of 2:4-dichlorophenoxyacetic acid by soil *Pseudomonas* species (Fernley, H. N. & Evans, W. C.) 22P
Chloroplasts, illuminated, coenzyme reduction by (Davenport, H. E.) 45P
Chloroplasts, isolated, destruction of crocin by (Friend, J. & Mayer, A. M.) 18P
Chlorpromazine, inhibition of electron transport between reduced diphosphopyridine nucleotide and cytochrome *c* by (Dawkins, M. J. R., Judah, J. D. & Rees, K. R.) 16
Cholesterol, interconversion of 7-dehydrocholesterol, lathosterol and, in rat (Mercer, E. I. & Glover, J.) 5P
Cholesterol, serum, effect of dietary linoleic acid on, in rat (Boyd, G. S. & Mawer, E. B.) 9P
*iso*Cholesterol in human-forearm sebum (Boughton, B. & Wheatley, V. R.) 144
Cholic acid, specific differential estimation of chenodeoxycholic acid and, in human plasma (Reid, R. H. P. & Boyd, G. S.) 9P
Choline acetylase, acyl-group specificity of (Berry, J. F. & Whittaker, V. P.) 447
[*Me*-^{14}C]Choline, incorporation of L-[^{14}C]serine and, into phospholipids of subcellular particles of rat liver (Dils, R. R. & Hübscher, G.) 26P
Choline sulphate, role of, in sulphur metabolism of fungi (Spencer, B. & Harada, T.) 34P
Cholinesterase in subcellular fractions from rat brain (Aldridge, W. N. & Johnson, M. K.) 270
Chromatophores, isolated, of *Rhodospirillum rubrum*, incorporation of [2-^{14}C]acetate and [2-^{14}C]mevalonate into unsaponifiable matter of (Davies, B. H. & Goodwin, T. W.) 10P
Chymotrypsin, reactions of, with tyrosine derivatives (Gutfreund, H. & Hammond, B. R.) 526
Cladophora rupestris, trans-β-glucosylation by extracts of (Duncan, W. A. M., Manners, D. J. & Thompson, J. L.) 295
Cod (*Gadus morrhua*), ubiquinone and other unsaponifiable constituents of (Pennock, J. F., Morton, R. A. & Lawson, D. E. M.) 4P
Coenzyme reduction by illuminated chloroplasts (Davenport, H. E.) 45P
Cortisone, diet and the action of, on protein metabolism (Goodlad, G. A. J. & Munro, H. N.) 343
Creatine phosphokinase and adenosine triphosphatase activity in muscles of exercised rats (Rawlinson, W. A. & Gould, M. K.) 44
Crithidia fasciculata, purification of vitamin B_{12}-active material from (Sanders, F. & Seaman, G. R.) 580
Crocin, destruction of, by isolated chloroplasts (Friend, J. & Mayer, A. M.) 18P
Cutting-grass (*Thryonomys swinderianus*), bile salts of (Haslewood, G. A. D. & Ogan, A. U.) 142
Cysteines, *S*-substituted, *N*-acetylation of, in rabbit, rat and guinea pig (Bray, H. G., Franklin, T. J. & James, S. P.) 465

Cytochrome b_2 and lactic dehydrogenase of baker's yeast (Appleby, C. A. & Morton, R. K.) 539

Dehydro*epi*androsterone in urine, estimation of (Fotherby, K.) 339
7-Dehydrocholesterol, interconversion of cholesterol, lathosterol and, in rat (Mercer, E. I. & Glover, J.) 5P
Dehydrogenases, intracellular distribution of, in rat-liver tissue (Beaufay, H., Bendall, D. S., Baudhuin, P. & de Duve, C.) 623
Density-gradient centrifuging, analysis of mitochondrial fractions from rat liver by (Beaufay, H., Bendall, D. S., Baudhuin, P., Wattiaux, R. & de Duve, C.) 628
11-Deoxycorticosterone, hydroxylation of, by adrenal glands of various mammals (Griffiths, K. & Grant, J. K.) 5P
Deoxyglucoses, production of phosphate esters by skin maintained on (Brooks, S. A., Lawrence, J. C. & Ricketts, C. R.) 566
Deoxyribonuclease, alkaline, intracellular distribution of, in rat-liver tissue (Beaufay, H., Bendall, D. S., Baudhuin, P. & de Duve, C.) 623
Deuterohaem, preparation and properties of myoglobin containing mesohaem and (Smith, M. H. & Gibson, Q. H.) 101
Deuterohaem, rate of combination of, with carbon monoxide (Smith, M. H.) 90
Diaphorase-like enzyme, soluble, isolation and purification of, from *Bacillus subtilis* (Kogut, M. & Lightbown, J. W.) 15P
2:4-Dichlorophenoxyacetic acid, metabolism of, by soil *Pseudomonas* species: isolation of α-chloromuconic acid as intermediate (Fernley, H. N. & Evans, W. C.) 22P
Digestive gland, arylsulphatase activity in the digestive juice and, of *Helix pomatia* (Dodgson, K. S. & Powell, G. M.) 666
Digestive gland, partial purification and properties of the arylsulphatase from, of *Helix pomatia* (Dodgson, K. S. & Powell, G. M.) 672
trans-1:2-Dihydro-1:2-dihydroxynaphthalene, conversion of naphthalene into compounds related to, by rabbits (Sims, P.) 389
3:4-Dimethylacetanilide, metabolism of, in rats (Boyland, E. & Sims, P.) 377
3:4-Dimethylaniline, metabolism of, in rats (Boyland, E. & Sims, P.) 377
2:4-Dinitrophenol, effect of, on membrane transport and intracellular phosphorylation of glucose in isolated rat heart (Morgan, H. E., Randle, P. J. & Regen, D. M.) 573
NN'-Diphenyl-*p*-phenylenediamine, cure of abnormal liability to haemolysis in vitamin E-deficient rats by (Moore, T. & Sharman, I. M.) 31P
Diphosphopyridine nucleotide (reduced)–cytochrome *c* reductase and coupled phosphorylation (Dawkins, M. J. R., Judah, J. D. & Rees, K. R.) 16
Diphosphopyridine nucleotide, reduced, soluble enzymes catalysing oxidation of, by molecular oxygen and hydrogen peroxide isolated from *Bacillus subtilis* (Lightbown, J. W. & Kogut, M.) 14P

Elastase, purification of proteolytic component of (Hall, D. A. & Czerkawski, J. W.) 356
Elastases, specificity of (Thomas, J. & Partridge, S. M.) 36P
Electron-spin resonance of xanthine oxidase solutions (Bray, R. C., Malmström, B. G. & Vänngård, T.) 193
Entodinium, carbohydrate fermentation by (Abou Akkada, A. R., Hobson, P. N. & Howard, B. H.) 44P
Epididymis, rat, properties of α-mannosidase and β-galactosidase from (Conchie, J. & Hay, A. J.) 327
Eremothecium ashbyii, threonine and biosynthesis of riboflavin in (Goodwin, T. W. & Horton, A. A.) 13P
Erythrocytes, effect of 'glucose cycloacetoacetate' on the regeneration of, and restoration of haemoglobin level in experimental anaemia in rats (Nath, M. C. & Prasannan, K. G.) 729
Escherichia coli, permeability of, to ribose and ribose nucleotides (Eggleston, L. V. & Krebs, H. A.) 264
Ester sulphate pattern of rat liver (Spencer, B.) 19P
Esterase in subcellular fractions from rat brain (Aldridge, W. N. & Johnson, M. K.) 270
Excitability in isolated cerebral tissues, loss of, and restoration by naturally occurring materials (Marks, N. & McIlwain, H.) 401
Exercise, biochemical adaptation as a response to (Gould, M. K. & Rawlinson, W. A.) 41; (Rawlinson, W. A. & Gould, M. K.) 44

Fatty acids, non-esterified, in sheep plasma (Lindsay, D. B.) 10P
Fatty acids, synthesis of, by the human red blood cell (James, A. T., Lovelock, J. E. & Webb, J. P. W.) 106
(+)-*anteiso*-Fatty acids, effect of administration of, on carcase fatty acid composition (Gerson, T., Shorland, F. B., Adams, Y. & Bell, M. E.) 594
Fluorescein–protein conjugates, investigation of unreacted fluorescent material in (Chadwick, C. S., Nairn, R. C. & McEntegart, M. G.) 41P
[^{14}C]Formate, changes in metabolism of DL-[β-^{14}C]serine and, in the vitamin B_{12}-deficient rat (Arnstein, H. R. V.) 23P
Formate metabolism in the vitamin B_{12}-deficient rat (Noronha, J. M. & Sreenivasan, A.) 732
Formic acid and pyruvic acid metabolism in *Zymosarcina ventriculi* (Bauchop, T. & Dawes, E. A.) 17P
Fumarate, yield of oxidative phosphorylation associated with the oxidation of succinate to (Greengard, P., Minnaert, K., Slater, E. C. & Betel, I.) 637
Fungi, role of choline sulphate in sulphur metabolism of (Spencer, B. & Harada, T.) 34P

β-Galactosidase from rat epididymis, properties of (Conchie, J. & Hay, A. J.) 327
Gas–liquid radiochromatography (Popják, G., Lowe, A. E., Moore, D., Brown, L. & Smith, F. A.) 33P
Gelatins and fractionated gelatins, distribution of mucoprotein in (Courts, A.) 600
Gelatins, fractionated, chain weight and rigidity relationship in (Courts, A.) 596
γ-Globulin, rabbit, hydrolysis of antibodies and, with crystalline papain (Porter, R. R.) 119
γ-Globulin, rabbit, ultracentrifugal examination of digestion products from (Charlwood, P. A.) 126
'Glucose cycloacetoacetate', effect of, on the regeneration of erythrocytes and restoration of haemoglobin level in experimental anaemia in rats (Nath, M. C. & Prasannan, K. G.) 729
Glucose, effects of insulin, anoxia, salicylate and 2:4-dinitrophenol on membrane transport and intracellular phosphorylation of, in isolated rat heart (Morgan, H. E., Randle, P. J. & Regen, D. M.) 573
Glucose, inhibitory effect of mepacrine on catabolism of, by *Plasmodium berghei* (Bowman, I. B. R., Grant, P. T., Kermack, W. O. & Ogston, D.) 42P
Glucose, production of phosphate esters by skin maintained on (Brooks, S. A., Lawrence, J. C. & Ricketts, C. R.) 566
Glucosylfructoses, synthesis of, by the action of a yeast α-glucosidase (Avigad, G.) 587
Glucuronide formation, mechanism of (Dutton, G. J.) 29P
Glutathione reductase in subcellular fractions from rat brain (Aldridge, W. N. & Johnson, M. K.) 270

L-α-Glycerophosphate oxidase and the terminal respiratory pathway of the bloodstream form of *Trypanosoma rhodesiense* (Grant, P. T. & Sargent, J. R.) 42P
Glycerylphosphorylcholine, separation of, from brain and spinal cord (Baker, R. W. R. & Porcellati, G.) 561
Glycerylphosphorylethanolamine, separation of, from brain and spinal cord (Baker, R. W. R. & Porcellati, G.) 561
Glycine, first metabolic pool of, in man (Watts, R. W. E. & Crawhall, J. C.) 277
Glycogen, liver, effect of diet and cortisone on (Goodlad, G. A. J. & Munro, H. N.) 343
Glycogen structure and *rigor mortis* in mammalian muscles (Lawrie, R. A., Manners, D. J. & Wright, A.) 485
Glycogen, structure of muscle-phosphorylase limit dextrins of (Walker, G. J. & Whelan, W. J.) 20P
Glyoxalase I reaction, mechanism of, and competitive inhibition by ophthalmic acid (Cliffe, E. E. & Waley, S. G.) 25P
Growth hormone, effect of treatment with, on incorporation of amino acids into liver proteins in a cell-free system (Korner, A.) 61
Gums, plum, composition of (Hough, L. & Pridham, J. B.) 550

Haem, enzymic formation of, by human and rat tissues (Lochhead, A. C. & Goldberg, A.) 39P
Haem, kinetics and equilibria in systems containing carbon monoxide, pyridine and (Smith, M. H.) 90
Haemoglobin, effect of 'glucose cycloacetoacetate' on the regeneration of erythrocytes and restoration of, in experimental anaemia in rats (Nath, M. C. & Prasannan, K. G.) 729
Haemolysis in vitamin E-deficient rats, cure of abnormal liability to, by *NN'*-diphenyl-*p*-phenylenediamine (Moore, T. & Sharman, I. M.) 31P
Heart, rat, isolated, effects of insulin, anoxia, salicylate and 2:4-dinitrophenol on membrane transport and intracellular phosphorylation of glucose in (Morgan, H. E., Randle, P. J. & Regen, D. M.) 573
Heart-muscle mitochondria, autonomic reversible swelling in (Beechey, R. B. & Holton, F. A.) 29P
Helix pomatia, arylsulphatase activity in the digestive juice and digestive gland of (Dodgson, K. S. & Powell, G. M.) 666
Helix pomatia, partial purification and properties of the arylsulphatase of the digestive gland of (Dodgson, K. S. & Powell, G. M.) 672
[^{14}C]Hexanoate, incorporation of, into milk constituents by perfused cow's udder (Lauryssens, M., Verbeke, R., Peeters, G. & Donck, A.) 71
Hexosamines, *N*-acetylation and estimation of (Levvy, G. A. & McAllan, A.) 127
Hippuric acid, concentration in urine of, in relation to 'first glycine metabolic pool' (Watts, R. W. E. & Crawhall, J. C.) 277
(*im*-DNP)Histidine, coloured reaction product from methoxycarbonyl chloride and (Carpenter, K. J., Jones, W. L. & Mason, E. L.) 11P
Histones as inhibitors of protein interactions and of metabolic response to stimulating agents in isolated cerebral tissues (McIlwain, H.) 514
Hyaline cartilage, distribution of polysaccharides in (Szirmai, J. A. & Doyle, J.) 35P
Hyaluronic acid in rooster comb, breakdown of (Doyle, J. & Szirmai, J. A.) 28P
Hydrogen peroxide, soluble enzymes catalysing oxidation of reduced diphosphopyridine nucleotide by molecular oxygen and, isolated from *Bacillus subtilis* (Lightbown, J. W. & Kogut, M.) 14P
Hydrolases, bound, enzymic release of (Beaufay, H. & de Duve, C.) 604
5-Hydroxyindoles, oxidation of serotonin and, during denaturation of oxyhaemoglobin (Blum, J. J. & Ling, N.-S.) 530
5-Hydroxylindolylaceturic acid, synthesis of (Keglević, D., Supek, Z., Kveder, S., Iskrić, S., Kečkeš, S. & Kisić, A.) 53
1-Hydroxy-2-naphthyl sulphate, formation of, in rabbits (Sims, P.) 389
5-Hydroxy[^{14}C]tryptamine, exogenous, metabolism of, in rats (Keglević, D., Supek, Z., Kveder, S., Iskrić, S., Kečkeš, S. & Kisić, A.) 53
Hypophysectomy of the rat, effect of, on incorporation of amino acids into liver proteins in a cell-free system (Korner, A.) 61

Inositol, effects of pH and temperature on reduction of periodates by (Sloane-Stanley, G. H. & Lock, P. A.) 8P
Insulin and insulin antagonists in protein fractions of normal human serum (Taylor, K. W., Vargas, L. & Randle, P. J.) 12P
Insulin, effect of, on incorporation of ^{14}C from ^{14}C-labelled carboxylic acids and bicarbonate into protein of isolated rat diaphragm (Manchester, K. L. & Krahl, M. E.) 39P
Insulin, effect of, on membrane transport and intracellular phosphorylation of glucose in isolated rat heart (Morgan, H. E., Randle, P. J. & Regen, D. M.) 573
Intestine, small, use of perfusion apparatus to study continuously the transport of ^{131}I-labelled iodide by (Acland, J. D.) 23P
Invertase, intestinal, rabbit (Carnie, J. A. & Porteous, J. W.) 48P
Invertases, kinetic properties of, obtained from different genera of sheep-rumen holotrich protozoa (Carnie, J. A. & Porteous, J. W.) 47P
Iodine, determination of, in biological material by activation analysis (Bowen, H. J. M.) 381
Iodine isotope (^{131}I), formation of thyroid hormones *in vivo* after administration of, as K^{131}I and of thyrotrophin (Feuer, G.) 349
Iodine isotope (^{131}I), localization of, in biosynthetically labelled [^{131}I]thyroxine (Plaskett, L. G.) 115
Iodine isotope (^{131}I), use of perfusion apparatus to study continuously the transport of, as iodide by small intestine (Acland, J. D.) 23P
Iodometry, spectrophotometric (Sloane-Stanley, G. H.) 8P
Iron, aconitase levels in leaves of mustard plants deficient in (Bacon, J. S. D., DeKock, P. C. & Palmer, M. J.) 41P
Iron, intracellular distribution of, in rat-liver tissue (Beaufay, H., Bendall, D. S., Baudhuin, P. & de Duve, C.) 623
Ischaemia, influence of, on the state of bound enzymes in rat liver (de Duve, C. & Beaufay, H.) 610
Isoniazid, metal-catalysed auto-oxidation of (Winder, F. G. & Denneny, J. M.) 500

Kidney, rat, synthesis of nicotinuric acid by preparations of (Jones, K. M. & Elliott, W. H.) 706

Lactic dehydrogenase and cytochrome b_2 of baker's yeast (Appleby, C. A. & Morton, R. K.) 539
Lactic dehydrogenase, effect of swimming on the level of, in muscles of young rats (Gould, M. K. & Rawlinson, W. A.) 41
Lathosterol, interconversion of cholesterol, 7-dehydrocholesterol and, in rat (Mercer, E. I. & Glover, J.) 5P
Lens, metabolism of xylose by, *in vivo* and *in vitro* (van Heyningen, R.) 197
DL-[1-^{14}C]Leucine, incorporation of [1-^{14}C]*iso*valeric acid and, into milk constituents by the perfused cow's udder (Verbeke, R., Lauryssens, M., Peeters, G. & James, A. T.) 24

Leucocidin, fractionation of, from *Staphylococcus aureus* (Woodin, A. M.) 225
Linoleic acid, dietary, effect of, on serum cholesterol in rat (Boyd, G. S. & Mawer, E. B.) 9P
Lipid biosynthesis in human blood *in vitro* (James, A. T., Lovelock, J. E. & Webb, J. P. W.) 106
Lipids, brain, crude, determination of neuraminic acid in (Long, C. & Staples, D. A.) 385
Lipoprotein of high lipid content from egg yolk (McIndoe, W. M.) 45P
Lipoproteins, plasma, lipid exchange between human red blood cells and (James, A. T., Lovelock, J. E. & Webb, J. P. W.) 106
Liquid scintillation-counting for [^{14}C]steroids (Stitch, S. R.) 287
Liver, incorporation of ^{65}Zn in subcellular fractions of, in mice after injection of zinc–glycine containing ^{65}Zn (Bartholomew, M. E., Tupper, R. & Wormall, A.) 256
Liver microsomes, synthesis of serum albumin by (Campbell, P. N. & Kernot, B. A.) 24P
Liver, ox, activation of an arylsulphatase from, by chloride and other anions (Webb, E. C. & Morrow, P. F. W.) 7
Liver, pig, amino acid-activating systems from (Finch, L. R. & Hele, P.) 11P
Liver, pigeon, conversion of malate into pyruvate by the malic enzyme of (Stickland, R. G.) 646
Liver, pigeon, synthesis of malate from pyruvate by the malic enzyme of (Stickland, R. G.) 654
Liver proteins, effect of hypophysectomy of the rat and of treatment with growth hormone on incorporation of amino acids into, in a cell-free system (Korner, A.) 61
Liver, rat, analysis of mitochondrial fractions from, by density-gradient centrifuging (Beaufay, H., Bendall, D. S., Baudhuin, P., Wattiaux, R. & de Duve, C.) 628
Liver, rat, ester sulphate pattern of (Spencer, B.) 19P
Liver, rat, incorporation of [*Me*-^{14}C]choline and L-[^{14}C]serine into phospholipids of subcellular particles of (Dils, R. R. & Hübscher, G.) 26P
Liver, rat, influence of ischaemia on the state of bound enzymes in (de Duve, C. & Beaufay, H.) 610
Liver, rat, influence of various hepatotoxic treatments on the state of bound enzymes in (Beaufay, H., van Campenhout, E. & de Duve, C.) 617
Liver, rat, intracellular distribution of some dehydrogenases, alkaline deoxyribonuclease and iron in (Beaufay, H., Bendall, D. S., Baudhuin, P. & de Duve, C.) 623
Liver, rat, vitamin A deficiency and ubiquinone and substance SC contents of (Morton, R. A. & Phillips, W. E. J.) 416
Liver, rat, vitamin A deficiency and ubiquinone and substance SC of (Morton, R. A. & Phillips, W. E. J.) 32P
Liver-enzyme preparations, formation of phosphorylated derivatives of mevalonic acid in (de Waard, A. & Popják, G.) 410
Lombricine, isolation of, and its enzymic phosphorylation (Pant, R.) 30
Lombricine, isolation of, and its possible biological precursor (Rosenberg, H. & Ennor, A. H.) 521
Lysolecithin, boundary electrophoresis of brain homogenates cleared by the action of (Gent, W. L. G.) 6P
Lysozyme, action of, on cell walls of Gram-positive microorganisms (Perkins, H. R.) 33P

Malate, conversion of, into pyruvate by the malic enzyme of pigeon liver (Stickland, R. G.) 646
Malate, synthesis of, from pyruvate by the malic enzyme of pigeon liver (Stickland, R. G.) 654
Malic dehydrogenase, effect of swimming on the level of, in muscles of young rats (Gould, M. K. & Rawlinson, W. A.) 41
Malic enzyme of pigeon liver, conversion of malate into pyruvate by (Stickland, R. G.) 646
Malic enzyme of pigeon liver, synthesis of malate from pyruvate by (Stickland, R. G.) 654
Mammary tumours, spontaneously occurring, incorporation of ^{65}Zn in subcellular fractions of, in mice after injection of zinc–glycine containing ^{65}Zn (Bartholomew, M. E., Tupper, R. & Wormall, A.) 256
α-Mannosidase from rat epididymis, properties of (Conchie, J. & Hay, A. J.) 327
Membrane transport, effects of insulin, anoxia, salicylate and 2:4-dinitrophenol on, and intracellular phosphorylation of glucose in isolated rat heart (Morgan, H. E., Randle, P. J. & Regen, D. M.) 573
Mepacrine, inhibitory effect of, on catabolism of glucose by *Plasmodium berghei* (Bowman, I. B. R., Grant, P. T., Kermack, W. O. & Ogston, D.) 42P
Mesohaem, preparation and properties of myoglobin containing deuterohaem and (Smith, M. H. & Gibson, Q. H.) 101
Mesohaem, rate of combination of, with carbon monoxide (Smith, M. H.) 90
Metal ions as precipitants for nucleic acids and their use in isolation of polynucleotides from leaves (Trim, A. R.) 298
Metal-catalysed auto-oxidation of isoniazid (Winder, F. G. & Denneny, J. M.) 500
Methoxycarbonyl chloride, coloured reaction product from (*im*-DNP)histidine and (Carpenter, K. J., Jones, W. L. & Mason, E. L.) 11P
(+)-14-Methylhexadecanoic acid, metabolism of, in the rat (Gerson, T., Shorland, F. B., Adams, Y. & Bell, M. E.) 594
(+)-12-Methyltetradecanoic acid, metabolism of, in the rat (Gerson, T., Shorland, F. B., Adams, Y. & Bell, M. E.) 594
[2-^{14}C]Mevalonate, incorporation of, into unsaponifiable matter of isolated chromatophores of *Rhodospirillum rubrum* (Davies, B. H. & Goodwin, T. W.) 10P
Mevalonic acid, formation of phosphorylated derivatives of, in liver-enzyme preparations (de Waard, A. & Popják, G.) 410
Microsomes, liver, synthesis of serum albumin by (Campbell, P. N. & Kernot, B. A.) 24P
Milk, incorporation of DL-[1-^{14}C]leucine and [1-^{14}C]*iso*valeric acid into constituents of, by the perfused cow's udder (Verbeke, R., Lauryssens, M., Peeters, G. & James, A. T.) 24
Milk, incorporation of sodium [1-^{14}C]hexanoate and sodium hydrogen [^{14}C]carbonate into constituents of, by perfused cow's udder (Lauryssens, M., Verbeke, R., Peeters, G. & Donck, A.) 71
Milk xanthine oxidase, oxidation of xanthine to uric acid catalysed by (Gutfreund, H. & Sturtevant, J. M.) 1
Mitochondria, heart-muscle, autonomic reversible swelling in (Beechey, R. B. & Holton, F. A.) 29P
Mitochondria, rat-brain, properties of adenosine triphosphatase of (Lagnado, J. R., Balázs, R. & Richter, D.) 18P
Monocotyledons, trisaccharide fraction of (Bacon, J. S. D.) 507
Mosquito, effect of 1:1:1-trichloro-2:2-di-(*p*-chlorophenyl)-ethane and related compounds on the adenosine triphosphate–phosphate exchange catalysed by a particulate fraction from (Gonda, O., Kaluszyner, A. & Avi-Dor, Y.) 583
Mucin, cervical, bovine, chemical properties of two mucoids from (Gibbons, R. A.) 209
Mucin, cervical, bovine, physicochemical properties of two mucoids from (Gibbons, R. A. & Glover, F. A.) 217
Mucoids from bovine cervical mucin, chemical properties of (Gibbons, R. A.) 209

Mucoids from bovine cervical mucin, physicochemical properties of (Gibbons, R. A. & Glover, F. A.) 217
Mucoprotein, distribution of, in gelatins and fractionated gelatins (Courts, A.) 600
Mucoprotein of cartilage, osmotic and chemical molecular weights of derivatives from (Partridge, S. M., Davis, H. F. & Adair, G. S.) 32P
Mung-bean seedling, enzymes of the pentose phosphate pathway in (Chakravorty, M. & Burma, D. P.) 48
Muscle, rabbit, kinetic studies on myokinase from (Callaghan, O. H. & Weber, G.) 473
Muscle, skeletal, purification and properties of thyroxine deiodinase from (Tata, J. R.) 36P
Muscle-phosphorylase limit dextrins, glycogen and amylopectin, structure of (Walker, G. J. & Whelan, W. J.) 20P
Muscles, mammalian, glycogen structure and *rigor mortis* in (Lawrie, R. A., Manners, D. J. & Wright, A.) 485
Muscles of exercised rats, adenosine triphosphatase and creatine phosphokinase activity in (Rawlinson, W. A. & Gould, M. K.) 44
Muscles of young rats, effect of swimming on the levels of lactic dehydrogenase, malic dehydrogenase and phosphorylase in (Gould, M. K. & Rawlinson, W. A.) 41
Mushroom tyrosinase, action of, on [16-^{14}C]oestrone (Jellinck, P. H.) 30P
Mustard plants, iron-deficient, aconitase levels in leaves of (Bacon, J. S. D., DeKock, P. C. & Palmer, M. J.) 41P
Myofilaments, uterus, properties of (Needham, D. M. & Williams, J. M.) 171
Myoglobin containing meso- and deutero-haem, preparation and properties of (Smith, M. H. & Gibson, Q. H.) 101
Myokinase, rabbit-muscle, kinetic studies on (Callaghan, O. H. & Weber, G.) 473

Naphthalene, conversion of, into compounds related to *trans*-1:2-dihydro-1:2-dihydroxynaphthalene by rabbits (Sims, P.) 389
Nerve, peripheral, phospholipid metabolism of, *in vivo* (Davison, A. N. & Dobbing, J.) 701
Nervous system, central, subcellular localization of transmitter substances in (Whittaker, V. P.) 37P
Neuraminic acid, determination of, in crude brain lipids (Long, C. & Staples, D. A.) 385
Nicotinamide, chromatographic method for estimation of (Jones, K. M.) 719
Nicotinuric acid, chromatographic method for estimation of (Jones, K. M.) 719
Nicotinuric acid synthesis, mechanism of (Jones, K. M.) 714
Nicotinuric acid, synthesis of, by rat-kidney preparations (Jones, K. M. & Elliott, W. H.) 706
Nitrobenzoic acids, reduction of nitro group of, by bacteria (Cartwright, N. J. & Cain, R. B.) 305
Nocardia, metabolism of nitrobenzoic acids by species of (Cartwright, N. J. & Cain, R. B.) 305
Nucleic acids in subcellular fractions from rat brain (Aldridge, W. N. & Johnson, M. K.) 270
Nucleic acids, metal ions as precipitants for, and their use in isolation and polynucleotides from leaves (Trim, A. R.) 298
Nucleic acids of bull semen (Bhargava, P. M., Bishop, M. W. H. & Work, T. S.) 242
Nucleolus, observations on functions of (Harris, H.) 362
Nucleotides, free, of bull semen (Bhargava, P. M., Bishop, M. W. H. & Work, T. S.) 242

Ochromonas malhamensis, assay of vitamin B_{12}-active material with (Sanders, F. & Seaman, G. R.) 580
[16-^{14}C]Oestrone, action of mushroom tyrosinase on (Jellinck, P. H.) 30P
Ophthalmic acid, mechanism of glyoxalase I reaction and competitive inhibition by (Cliffe, E. E. & Waley, S. G.) 25P
Ovalbumin, changes in optical rotation, extinction and viscosity of, during serial denaturation in solutions of urea (Steven, F. S. & Tristram, G. R.) 86
Oxaloacetate-synthesizing enzyme, properties of (Stickland, R. G.) 660
Oxidative phosphorylation, yield of, associated with the oxidation of succinate to fumarate (Greengard, P., Minnaert, K., Slater, E. C. & Betel, I.) 637
Oxygen, molecular, soluble enzymes catalysing oxidation of reduced diphosphopyridine nucleotide by hydrogen peroxide and, isolated from *Bacillus subtilis* (Lightbown, J. W. & Kogut, M.) 14P
Oxyhaemoglobin, oxidation of serotonin and 5-hydroxyindoles during denaturation of (Blum, J. J. & Ling, N.-S.) 530

Papain, crystalline, hydrolysis of rabbit γ-globulin and antibodies with (Porter, R. R.) 119
Parapepsins associated with porcine pepsin with proteolytic activity (Ryle, A. P. & Porter, R. R.) 75
Pentose cycle, arteriovenous $^{14}CO_2$ differences and, in cow's udder (Wood, H. G., Gillespie, R., Hansen, R. G., Wood, W. A. & Hardenbrook, H. J.) 694
Pentose phosphate pathway, enzymes of, in mung-bean seedling (Chakravorty, M. & Burma, D. P.) 48
Pepsin, porcine, two proteolytic enzymes associated with (Ryle, A. P. & Porter, R. R.) 75
Pepsin-catalysed transpeptidation of the amino-transfer type (Neumann, H., Levin, Y., Berger, A. & Katchalski, E.) 33
Peptide component, site of synthesis of, of cell wall of *Bacillus megaterium* (Brookes, P., Crathorn, A. R. & Hunter, G. D.) 396
Periodates, effects of pH and temperature on reduction of, by inositol (Sloane-Stanley, G. H. & Lock, P. A.) 8P
Phenols, observations on the metabolic fate of sulphate esters of (Dodgson, K. S. & Tudball, N.) 27P
Phosphate esters, nitrogen-containing, separation of, from brain and spinal cord by ion-exchange chromatography (Baker, R. W. R. & Porcellati, G.) 561
Phosphate esters of mammalian skin maintained on glucose and various deoxyglucoses (Brooks, S. A., Lawrence, J. C. & Ricketts, C. R.) 566
Phosphate exchange, effect of 1:1:1-trichloro-2:2-di-(*p*-chlorophenyl)ethane and related compounds on, catalysed by a particulate fraction from the mosquito (Gonda, O., Kaluszyner, A. & Avi-Dor, Y.) 583
Phosphate, incorporation of, into blood-cell phospholipids (Rowe, C. E.) 438
Phospholipid, brain and peripheral nerve, metabolism of, *in vivo* (Davison, A. N. & Dobbing, J.) 701
Phospholipids, biosynthesis of, by human blood cells (Rowe, C. E.) 438
Phospholipids in human aorta (Smith, E. B.) 34P
Phospholipids of subcellular particles of rat liver, incorporation of [*Me*-^{14}C]choline and L-[^{14}C]serine into (Dils, R. R. & Hübscher, G.) 26P
Phosphomevalonic acid, formation of, by liver enzymes (de Waard, A. & Popják, G.) 410
Phosphoprotein, intracellular localization in brain of, involved in metabolic response of cortical slices to electrical stimulation (Heald, P. J.) 132
Phosphorylase, effect of swimming on the level of, in muscles of young rats (Gould, M. K. & Rawlinson, W. A.) 41
Phosphorylation, coupled, and reduced diphosphopyridine nucleotide–cytochrome *c* reductase (Dawkins, M. J. R., Judah, J. D. & Rees, K. R.) 16

Phosphorylation, enzymic, and isolation of lombricine (Pant, R.) 30
Phosphorylcholine, separation of, from brain and spinal cord (Baker, R. W. R. & Porcellati, G.) 561
Phosphorylethanolamine, separation of, from brain and spinal cord (Baker, R. W. R. & Porcellati, G.) 561
Phosphorylserine, separation of, from brain and spinal cord (Baker, R. W. R. & Porcellati, G.) 561
Phthalic acid, metabolic pathway of, by soil *Pseudomonas* species (Ribbons, D. W. & Evans, W. C.) 20P
Pituitary gland, effect of, on protein biosynthesis (Korner, A.) 61
Plasma, human, specific differential estimation of cholic acid and chenodeoxycholic acid in (Reid, R. H. P. & Boyd, G. S.) 9P
Plasma lipoproteins, fatty acid composition of (James, A. T., Lovelock, J. E. & Webb, J. P. W.) 106
Plasma, sheep, non-esterified fatty acids in (Lindsay, D. B.) 10P
Plasmodium berghei, inhibitory effect of mepacrine on catabolism of glucose by (Bowman, I. B. R., Grant, P. T., Kermack, W. O. & Ogston, D.) 42P
Plum gums, composition of (Hough, L. & Pridham, J. B.) 550
Polyamines, inhibition of bacteriophage multiplication by proflavine and reversal by (Kay, D.) 149
Polynucleotides, metal ions as precipitants for nucleic acids and their use in isolation of, from leaves (Trim, A. R.) 298
Polysaccharides, distribution of, in hyaline cartilage (Szirmai, J. A. & Doyle, J.) 35P
Porphobilinogen deaminase, partial purification and properties of, from *Rhodopseudomonas spheroides* (Hoare, D. S. & Heath, H.) 679
Potassium ions, inhibition of oxaloacetate-synthesizing enzyme by (Stickland, R. G.) 660
Potato, sprouting, properties of the starch of the tuber and shoots of (Banks, W. & Greenwood, C. T.) 237
Proflavine, inhibition of bacteriophage multiplication by, and reversal by certain polyamines (Kay, D.) 149
Protein interactions and metabolic response to stimulating agents in isolated cerebral tissues, histones as inhibitors of (McIlwain, H.) 514
Protein interactions which condition metabolic response to electrical pulses in mammalian cerebral tissues (McIlwain, H.) 30P
Protein, liver, effect of cortisone on (Goodlad, G. A. J. & Munro, H. N.) 343
Protein metabolism, diet and action of cortisone on (Goodlad, G. A. J. & Munro, H. N.) 343
Protein of isolated rat diaphragm, effect of insulin on incorporation of ^{14}C from ^{14}C-labelled carboxylic acids and bicarbonate into (Manchester, K. L. & Krahl, M. E.) 39P
Protein, spectrophotometric determination of, at 210 mμ (Tombs, M. P., Souter, F. & Maclagan, N. F.) 167
Protein synthesis, intermediate reactions in, by isolated cytoplasmic-membrane fraction of *Bacillus megaterium* (Hunter, G. D., Brookes, P., Crathorn, A. R. & Butler, J. A. V.) 369
Proteins of bull spermatozoa, incorporation of [^{14}C]amino acids into (Bhargava, P. M., Bishop, M. W. H. & Work, T. S.) 247
Protocatechuic acid, metabolic pathway of, by *Pseudomonas* species (Ribbons, D. W. & Evans, W. C.) 21P
Protohaem, rate of combination of, with carbon monoxide (Smith, M. H.) 90
Protozoa, holotrich, sheep-rumen, kinetic properties of invertases obtained from different genera of (Carnie, J. A. & Porteous, J. W.) 47P
Pseudomonas species, pathway of metabolism of protocatechuic acid by (Ribbons, D. W. & Evans, W. C.) 21P
Pseudomonas species, soil, metabolism of 2:4-dichlorophenoxyacetic acid by (Fernley, H. N. & Evans, W. C.) 22P
Pseudomonas species, soil, pathway of metabolism of phthalic acid by (Ribbons, D. W. & Evans, W. C.) 20P
Putrescine, reversal of proflavine inhibition of bacteriophage multiplication by (Kay, D.) 149
Pyridine haemochromogen, mechanism of formation of (Smith, M. H.) 90
Pyridine, kinetics and equilibria in systems containing haem, carbon monoxide and (Smith, M. H.) 90
Pyridine nucleotides, reduced and oxidized, determination of, in animal tissues (Bassham, J. A., Birt, L. M., Hems, R. & Loening, U. E.) 491
Pyruvate, conversion of malate into, by the malic enzyme of pigeon liver (Stickland, R. G.) 646
Pyruvate, synthesis of malate from, by the malic enzyme of pigeon liver (Stickland, R. G.) 654
Pyruvic acid and formic acid metabolism in *Zymosarcina ventriculi* (Bauchop, T. & Dawes, E. A.) 17P

Quinones, chemistry and biosynthesis of, in micro-organisms (Birkinshaw, J. H.) 1P
Quinones in electron transport and oxidative phosphorylation (Redfearn, E. R. & Pumphrey, A. M.) 3P
Quinones, naturally occurring, distribution and nature of (Thomson, R. H.) 1P

Rana catesbeiana, thyroxine and visual pigment conversion in larvae of (Wilt, F. H.) 13P
Rhodopseudomonas spheroides, partial purification and properties of porphobilinogen deaminase and uroporphyrinogen decarboxylase from (Hoare, D. S. & Heath, H.) 679
Rhodospirillum rubrum, incorporation of [2-^{14}C]acetate and [2-^{14}C]mevalonate into unsaponifiable matter of isolated chromatophores of (Davies, B. H. & Goodwin, T. W.) 10P
Riboflavin and threonine biosynthesis in *Eremothecium ashbyii* (Goodwin, T. W. & Horton, A. A.) 13P
Ribonuclease, inactivation of, during isolation of ribonucleic acids and ribonucleoproteins from yeast (Brownhill, T. J., Jones, A. S. & Stacey, M.) 434
Ribonucleic acid, liver, effect of cortisone on (Goodlad, G. A. J. & Munro, H. N.) 343
Ribonucleic acid, nuclear and cytoplasmic, turnover of, in two types of animal cell (Harris, H.) 362
Ribonucleic acids, inactivation of ribonuclease during isolation of, from yeast (Brownhill, T. J., Jones, A. S. & Stacey, M.) 434
Ribonucleoprotein fraction of microsomes, incorporation of amino acids into (Cohn, P.) 25P
Ribonucleoproteins, inactivation of ribonuclease during isolation of, from yeast (Brownhill, T. J., Jones, A. S. & Stacey, M.) 434
Ribose nucleotides, permeability of *Escherichia coli* to ribose and (Eggleston, L. V. & Krebs, H. A.) 264
Ribose, permeability of *Escherichia coli* to ribose nucleotides and (Eggleston, L. V. & Krebs, H. A.) 264
Rigor mortis and glycogen structure in mammalian muscles (Lawrie, R. A., Manners, D. J. & Wright, A.) 485
Rumen contents, sheep, effect of, on triglycerides *in vitro* (Garton, G. A., Lough, A. K. & Vioque, E.) 46P

Salicylate, effect of, on membrane transport and intracellular phosphorylation of glucose in isolated rat heart (Morgan, H. E., Randle, P. J. & Regen, D. M.) 573
Sarcosine, controlled biosynthesis of actinomycin with (Katz, E. & Goss, W. A.) 458
Sebum, human-forearm, composition of unsaponifiable matter of (Boughton, B. & Wheatley, V. R.) 144

Semen, bull, chemical composition of, with special reference to nucleic acids, free nucleotides and free amino acids (Bhargava, P. M., Bishop, M. W. H. & Work, T. S.) 242
DL-[β-^{14}C]Serine, changes in metabolism of [^{14}C]formate and, in vitamin B_{12}-deficient rat (Arnstein, H. R. V.) 23P
L-[^{14}C]Serine, incorporation of [*Me*-^{14}C]choline and, into phospholipids of subcellular particles of rat liver (Dils, R. R. & Hübscher, G.) 26P
Serotonin, oxidation of 5-hydroxyindoles and, during denaturation of oxyhaemoglobin (Blum, J. J. & Ling, N.-S.) 530
Serum, effect of, on restoration of loss of excitability in isolated cerebral tissues (Marks, N. & McIlwain, H.) 401
Serum, human, insulin and insulin antagonists in protein fractions of (Taylor, K. W., Vargas, L. & Randle, P. J.) 12P
Shikimic acid, phosphorylation of, by ultrasonic extracts of micro-organisms (Fewster, J. A.) 14P
Sialic acid, presence of, in connective tissues (Courts, A.) 596
Silver, detection of carbohydrates with, in the presence of borate (Britton, H. G.) 19P
Skin, mammalian, phosphate esters of, maintained on glucose and various deoxyglucoses (Brooks, S. A., Lawrence, J. C. & Ricketts, C. R.) 566
Sodium [1-^{14}C]hexanoate, incorporation of, into milk constituents by perfused cow's udder (Lauryssens, M., Verbeke, R., Peeters, G. & Donck, A.) 71
Sodium hydrogen [^{14}C]carbonate, incorporation of, into milk constituents by perfused cow's udder (Lauryssens, M., Verbeke, R., Peeters, G. & Donck, A.) 71
Sodium salicylate, stabilization of xanthine oxidase by (Bergel, F. & Bray, R. C.) 182
Spectrophotometric iodometry, aspects of (Sloane-Stanley, G. H.) 8P
Spermatozoa, bull, chemical composition of (Bhargava, P. M., Bishop, M. W. H. & Work, T. S.) 242
Spermatozoa, bull, incorporation of [^{14}C]amino acids into proteins of (Bhargava, P. M., Bishop, M. W. H. & Work, T. S.) 247
Sphingomyelin, brain, observations on metabolism of (Ansell, G. B. & Spanner, S.) 3P
Spinal cord, separation of nitrogen-containing phosphate esters from brain and, by ion-exchange chromatography (Baker, R. W. R. & Porcellati, G.) 561
Staphylococcus aureus, differential effect of arginine and canavanine on growth and enzyme formation in (Richmond, M. H.) 155
Staphylococcus aureus, fractionation of a leucocidin from (Woodin, A. M.) 225
Staphylococcus aureus, incorporation of canavanine by (Richmond, M. H.) 261
Starch of the tuber and shoots of sprouting potato, properties of (Banks, W. & Greenwood, C. T.) 237
[^{14}C]Steroids, liquid scintillation-counting for (Stitch, S. R.) 287
Sterol, influence of vitamin E status on (Morton, R. A. & Phillips, W. E. J.) 427
Strandin, determination of, in brain lipids (Long, C. & Staples, D. A.) 385
Substance SC and ubiquinone contents of rat liver in vitamin A deficiency (Morton, R. A. & Phillips, W. E. J.) 416
Substance SC and ubiquinone of rat liver in vitamin A deficiency (Morton, R. A. & Phillips, W. E. J.) 32P
Substance SC, content of, in tissues in relation to vitamin K status (Morton, R. A. & Phillips, W. E. J.) 421
Substance SC in liver of vitamin A-deficient rats, effect of adrenalectomy on (Phillips, W. E. J. & Morton, R. A.) 430
Succinate, yield of oxidative phosphorylation associated with the oxidation of, to fumarate (Greengard, P., Minnaert, K., Slater, E. C. & Betel, I.) 637
Succinate–neotetrazolium reductase, activation of, by vitamin K_3 (Slater, T. F.) 314
Succinic dehydrogenase in subcellular fractions from rat brain (Aldridge, W. N. & Johnson, M. K.) 270
Sucrose, derivatives of, in monocotyledons (Bacon, J. S. D.) 507
Sulphate esters of phenols, observations on the metabolic fate of (Dodgson, K. S. & Tudball, N.) 27P
Sulphur metabolism of fungi, role of choline sulphate in (Spencer, B. & Harada, T.) 34P
Swimming, effect of, on the levels of lactic dehydrogenase, malic dehydrogenase and phosphorylase in muscles of young rats (Gould, M. K. & Rawlinson, W. A.) 41

Taurochenodeoxycholic acid in the bile of the cutting-grass (*Thryonomys swinderianus*) (Haslewood, G. A. D. & Ogan, A. U.) 142
Taurodeoxycholic acid in the bile of the cutting-grass (*Thryonomys swinderianus*) (Haslewood, G. A. D. & Ogan, A. U.) 142
Tetrahymena pyriformis, trans-α-glucosylation by extracts of (Archibald, A. R. & Manners, D. J.) 292
Tetraiodothyronine, iodide excretion and oxygen consumption by rats after injection of (Wilkinson, J. H.) 334
Threonine and riboflavin biosynthesis in *Eremothecium ashbyii* (Goodwin, T. W. & Horton, A. A.) 13P
Thyroid hormones, formation of, *in vivo* after administration of K^{131}I and thyrotrophin (Feuer, G.) 349
Thyrotrophin, formation of thyroid hormones *in vivo* after administration of K^{131}I and (Feuer, G.) 349
Thyroxine, acetic acid analogues of tri-iodothyronine and (Wilkinson, J. H.) 334
Thyroxine and visual pigment conversion in larvae of *Rana catesbeiana* (Wilt, F. H.) 13P
[^{131}I]Thyroxine, biosynthetically labelled, localization of ^{131}I in (Plaskett, L. G.) 115
Thyroxine deiodinase from skeletal muscle, purification and properties of (Tata, J. R.) 36P
Tomato, determination of halogens in seeds of (Bowen, H. J. M.) 381
Trans-β-glucosylation by extracts of *Cladophora rupestris* and *Ulva lactuca* (Duncan, W. A. M., Manners, D. J. & Thompson, J. L.) 295
Trans-α-glucosylation by extracts of *Tetrahymena pyriformis* (Archibald, A. R. & Manners, D. J.) 292
Transmitter substances, subcellular localization of, in the central nervous system (Whittaker, V. P.) 37P
Transpeptidation, pepsin-catalysed, of the amino-transfer type (Neumann, H., Levin, Y., Berger, A. & Katchalski, E.) 33
1:1:1-Trichloro-2:2-di-(*p*-chlorophenyl)ethane, effect of related compounds and, on the adenosine triphosphate–phosphate exchange catalysed by a particulate fraction from the mosquito (Gonda, O., Kaluszyner, A. & Avi-Dor, Y.) 583
Triglycerides, effect of sheep-rumen contents on, *in vitro* (Garton, G. A., Lough, A. K. & Vioque, E.) 46P
Tri-iodothyronine, acetic acid analogues of thyroxine and (Wilkinson, J. H.) 334
Tri-iodothyronine, iodide excretion and oxygen consumption by rats after injection of (Wilkinson, J. H.) 334
Triphosphopyridine nucleotide, oxidation of, in the presence of metal ions and sulphite (Stickland, R. G.) 654
Trisaccharide fraction of some monocotyledons (Bacon, J. S. D.) 507

Trypanosoma rhodesiense, L-α-glycerophosphate oxidase and the terminal respiratory pathway of the bloodstream form of (Grant, P. T. & Sargent, J. R.) 42P
Tumour, incorporation of ^{65}Zn in subcellular fractions of, of mice after injection of zinc–glycine containing ^{65}Zn (Bartholomew, M. E., Tupper, R. & Wormall, A.) 256
Tyrosinase, mushroom, action of, on [16-^{14}C]oestrone (Jellinck, P. H.) 30P
Tyrosine derivatives, reactions of chymotrypsin with (Gutfreund, H. & Hammond, B. R.) 526
L-Tyrosine *O*-sulphate, ^{35}S-labelled, synthesis of, by tissues *in vivo* and *in vitro* (Grimes, A. J.) 723

Ubiquinone and other unsaponifiable constituents of cod (*Gadus morrhua*) (Pennock, J. F., Morton, R. A. & Lawson, D. E. M.) 4P
Ubiquinone and substance SC contents of rat liver in vitamin A deficiency (Morton, R. A. & Phillips, W. E. J.) 416
Ubiquinone and substance SC of rat liver in vitamin A deficiency (Morton, R. A. & Phillips, W. E. J.) 32P
Ubiquinone, content of, in tissues in relation to vitamin K status (Morton, R. A. & Phillips, W. E. J.) 421
Ubiquinone in liver of rats deficient in vitamin A, effect of adrenalectomy on (Phillips, W. E. J. & Morton, R. A.) 430
Ubiquinone in rats deficient in vitamin A (Moore, T. & Sharman, I. M.) 6P
Ubiquinone, influence of vitamin E status on (Morton, R. A. & Phillips, W. E. J.) 427
Ubiquinones (Morton, R. A.) 2P
Udder, cow's, arteriovenous $^{14}CO_2$ differences and the pentose cycle in (Wood, H. G., Gillespie, R., Hansen, R. G., Wood, W. A. & Hardenbrook, H. J.) 694
Udder, cow's, perfused, incorporation of DL-[1-^{14}C]leucine and [1-^{14}C]*iso*valeric acid into milk constituents by (Verbeke, R., Lauryssens, M., Peeters, G. & James, A. T.) 24
Udder, cow's, perfused, incorporation of sodium [1-^{14}C]-hexanoate and sodium hydrogen [^{14}C]carbonate into milk constituents by (Lauryssens, M., Verbeke, R., Peeters, G. & Donck, A.) 71
Ulva lactuca, trans-β-glucosylation by extracts of (Duncan, W. A. M., Manners, D. J. & Thompson, J. L.) 295
Urea, changes in optical rotation, extinction and viscosity of ovalbumin during serial denaturation in solutions of (Steven, F. S. & Tristram, G. R.) 86
Uric acid, oxidation of xanthine to, catalysed by milk xanthine oxidase (Gutfreund, H. & Sturtevant, J. M.) 1
Urine, content of amines in (Asatoor, A. M. & Dalgliesh, C. E.) 26P
Urine, estimation of dehydro*epi*androsterone in (Fotherby, K.) 339
Uroporphyrinogen decarboxylase, partial purification and properties of, from *Rhodopseudomonas spheroides* (Hoare, D. S. & Heath, H.) 679
Uterus actomyosin and myofilaments, properties of (Needham, D. M. & Williams, J. M.) 171

[1-^{14}C]*iso*Valeric acid, incorporation of DL-[1-^{14}C]leucine and, into milk constituents by the perfused cow's udder (Verbeke, R., Lauryssens, M., Peeters, G. & James, A. T.) 24
Visual pigment and thyroxine conversion in larvae of *Rana catesbeiana* (Wilt, F. H.) 13P
Vitamin A, content of, in tissues in relation to vitamin K status (Morton, R. A. & Phillips, W. E. J.) 421
Vitamin A deficiency, ubiquinone and substance SC contents of rat liver in (Morton, R. A. & Phillips, W. E. J.) 416, 32P
Vitamin A, ubiquinone in rats deficient in (Moore, T. & Sharman, I. M.) 6P
Vitamin B_{12}, formate metabolism in rats deficient in (Noronha, J. M. & Sreenivasan, A.) 732
Vitamin B_{12}-active material from *Crithidia fasciculata*, purification of (Sanders, F. & Seaman, G. R.) 580
Vitamin E, unsaponifiable constituents of liver, kidney and heart tissues from rats deficient in (Morton, R. A. & Phillips, W. E. J.) 427
Vitamin K status, unsaponifiable constituents of rat tissues in relation to (Morton, R. A. & Phillips, W. E. J.) 421
Vitamin K_3, activation of succinate–neotetrazolium reductase by (Slater, T. F.) 314

Xanthine, milk xanthine oxidase and oxidation of, to uric acid (Gutfreund, H. & Sturtevant, J. M.) 1
Xanthine oxidase, milk, oxidation of xanthine to uric acid catalysed by (Gutfreund, H. & Sturtevant, J. M.) 1
Xanthine oxidase, problems of enzyme inactivation and stabilization of (Bergel, F. & Bray, R. C.) 182
Xanthine oxidase solutions, electron-spin resonance of (Bray, R. C., Malmström, B. G. & Vänngård, T.) 193
Xanthine oxidase, variation in stability and the presence of an inhibitor in certain preparations of (Bray, R. C.) 690
Xylose, metabolism of, by lens *in vivo* and *in vitro* (van Heyningen, R.) 197

Yeast, baker's, lactic dehydrogenase and cytochrome b_2 of (Appleby, C. A. & Morton, R. K.) 539
Yeast α-glucosidase, synthesis of glucosylfructoses by the action of (Avigad, G.) 587
Yeast, inactivation of ribonuclease during isolation of ribonucleic acids and ribonucleoproteins from (Brownhill, T. J., Jones, A. S. & Stacey, M.) 434
Yolk, egg, lipoprotein of high lipid content from (McIndoe, W. M.) 45P

Zinc, inhibition of yeast ribonuclease by (Brownhill, T. J., Jones, A. S. & Stacey, M.) 434
Zinc isotope (^{65}Zn), incorporation of, into subcellular fractions of liver and spontaneously occurring mammary tumours of mice after injection of zinc–glycine containing ^{65}Zn (Bartholomew, M. E., Tupper, R. & Wormall, A.) 256
Zymosarcina ventriculi, pyruvic acid and formic acid metabolism in (Bauchop, T. & Dawes, E. A.) 17P

THE BIOCHEMICAL JOURNAL

NOTES FOR CONTRIBUTORS

Papers submitted for publication in the *Biochemical Journal* should be written concisely. The sections below concerning the preparation of the typescript give only general indications. Authors are urged to consult the more detailed *Suggestions to Authors, Chemical Nomenclature and Abbreviations, Symbols, Usages and Conventions,* which was published in May 1957 (*Biochem. J.* 1957, 66, 1); also *Notes on Preparation of Illustrations,* which was published in January 1956 (*Biochem. J.* 1956, 62). Copies of these two pamphlets may be obtained from the Editorial Office (*Biochemical Journal*), The Lister Institute, Chelsea Bridge Road, London, S.W. 1, price 1*s.* 6*d.* (*Suggestions to Authors*) and 1*s.* (*Notes on Preparation of Illustrations*) post free.

Strict observance of the requirements of the *Biochemical Journal* will shorten the period between the receipt of a paper and its publication.

Communications. Papers submitted for publication should be sent to the Secretary to the Editorial Board (*Biochemical Journal*), The Lister Institute of Preventive Medicine, Chelsea Bridge Road, London, S.W. 1.

Abstracts. Authors should submit with their typescript an abstract suitable for inclusion in *International Abstracts of Biological Sciences.* This abstract will not appear in the *Biochemical Journal* but will be edited before being passed for publication in the *Abstracts.*

The abstract should outline as briefly as possible the results and definitive conclusions of the work submitted. Details of methods are generally not required. A paper of average length should be abstracted in about 100 words. The abstract should be typed in double spacing on a separate quarto sheet in the following form: title; name(s) of author(s); *Biochem. J.* (space for year, volume and page reference); address (for reprint applications); abstract. For example:

> The metabolism of short-chain fatty acids in the sheep. 4. The pathway of propionate metabolism in rumen epithelial tissue. R. J. Pennington and T. M. Sutherland. *Biochem. J.* 1956, **63**, 618–628 (Rowett Research Institute, Bucksburn, Aberdeenshire, Scotland)—(Abstract).

Reprints. Where at least one author of a paper is a member of the Biochemical Society, twenty-five reprints are supplied free of cost. An author may purchase additional reprints if he notifies the Press on the appropriate form immediately the proof of the paper is received. Communications about the purchase of reprints should be addressed to the University Press, Cambridge.

General. It is the policy of the *Biochemical Journal* to publish papers in all fields of biochemistry—plant, animal and microbiological—provided that they describe results which make a new and fundamental contribution to biochemical knowledge, or that they describe methods applicable to biochemical problems. Submission of a paper to the Editorial Board will be held to imply that it reports unpublished work, that it is not under consideration for publication elsewhere, and that if accepted for the *Biochemical Journal* it will not be published elsewhere in the same form, either in English or in any other language, without the consent of the Editorial Board.

Contributors who reside abroad may nominate somebody in Great Britain who is willing to correct the proofs of their papers. Proofs are also sent to all authors residing abroad, if necessary by airmail, whether or not they have nominated a proof reader

p.t.o.

in Great Britain; if these are returned immediately, it will normally be possible to incorporate corrections in the final proof. The method of correcting proofs given in B.S. 1219 or B.S. 1219C (obtainable from the British Standards Institution) is preferred.

Papers should be headed by an informative title, by the names of the authors and by the name and address of the Laboratory where the work was performed. Female authors should use one given name and the surname, and male authors should use initials and surname only. Descriptive material about the author, e.g. Beit Memorial Fellow, or details of financial support, should appear as a footnote on the first page or, preferably, in the acknowledgements at the end of the paper.

Typescripts should bear the name and address of the person to whom the proof of the paper is to be sent, and should give also a shortened version of the title, not exceeding forty-five letters and spaces in length, suitable for a running title in the published pages of the work.

If a paper that has been returned to an author for revision is not resubmitted within six months, it will be deemed to be a new paper and the date of receipt altered accordingly. A revised paper containing a significant amount of new material will also be redated.

A paper should be written only when a piece of work is rounded off. Preliminary or abortive experiments should not be described.

It would help the editors if the author, when submitting a paper which is part of a series would enclose reprints of the immediately preceding parts.

Form of papers submitted for publication. Papers should be in double-spaced typing on sheets of uniform size with wide margins. Top copies only should be submitted. The paper should be written in English.

The onus of preparing a paper in a form suitable for sending to press lies in the first place with the author, who should first consult the detailed *Suggestions to Authors, Chemical Nomenclature and Abbreviations, Symbols, Usages and Conventions.* Authors should also refer to a current issue of the *Journal* in order to make themselves familiar with the typographical conventions, use of cross-headings, lay-out of tables, citation of references, etc. The need for editorial revision of a badly prepared typescript will lead to delay in publication. Papers on specialized subjects should be presented so that they are intelligible to the ordinary reader of the *Journal*. Sufficient information should be included to permit repetition of the experimental work.

Generally, papers should be divided clearly into sections, as follows: (*a*) introduction, containing the reasons for doing the work; (*b*) Experimental methods: with chemical papers the experimental part may appear towards the end, but otherwise should follow the introduction; (*c*) Results: these should be given concisely; the use of both tables and figures to illustrate the same results will only rarely be permitted; only illustrative protocols should be included; (*d*) Discussion: it is desirable that the presentation of the results should be separated from the discussion of their significance; this section should be strictly limited to discussion, and should not recapitulate results; (*e*) a Summary, about 3% of the length of the paper: the paragraphs of the Summary should be numbered; (*f*) acknowledgements; (*g*) References. The arrangement suggested for sections (*b*)–(*d*) is not binding on authors; other ways of arrangement are sometimes more suitable.

The *Biochemical Journal* uses as a standard for spelling the *Concise Oxford Dictionary of Current English* (Oxford: Clarendon Press).

Illustrations. Diagrams that do not conform with the directions given in *Notes on Preparation of Illustrations* may have to be redrawn by the Press and the expense charged to the author. Legends and captions should be written so that the general meaning of each illustration can be understood without reference to the text, and so that the exact experimental conditions used to obtain the results illustrated are made clear. Illustrations requiring reproduction as half-tone plates should be avoided whenever possible. Photographs or drawings of paper chromatograms, particularly one-dimensional, are not generally published.

Tables. Tables should have headings which make their general meaning comprehensible without reference to the text. Conditions specific to the particular experiment should be stated. Reference to the text for general experimental methods is permissible provided that there is no ambiguity. The units in which the results are expressed, e.g. g./100 ml., should be given at the top of each column, and not repeated on each line of the table.

Tables should be typed on separate sheets and their approximate position in the text indicated. Words or numerals should be repeated on successive lines: 'ditto' or ',,' are not to be used.

Footnotes. These should be avoided in the text as far as possible.

NEW I·D·L range of laboratory counting equipment

'1800 SERIES'

These low-priced, space-saving units give full operational accuracy, flexibility in application, and simplicity of maintenance. Details of the first three units – the Scaler Type 1800, H.V. Supply Type 1820, and Amplifier/Selector Type 1830 are given here. *Watch for details* of the Pre-scaler 1850, Ratemeter 1810, Potentiometer 1840, and Timer Unit 1860.

THE '1830' AMPLIFIER-SELECTOR

Amplifier: Gain: x 5: x 200: x 1,000.

Input Impedance: 1 MΩ

Rise Time: 250 mμ sec.

Stability: 1%

Discriminator/Selector:

Threshold: (variable) 5 to 50 V.

Stability: ± 0.25 V.

Channel Width: (variable) 1 – 50 V.

Stability: ± 0.25 V.

THE '1820' H.V. SUPPLY

Voltage and Current output: (Positive only).

Range 1: 250 to 1,000 volts. 0.3 mA.

Range 2: 500 to 2,000 volts. 0.1 mA.

Stability: (For Mains change +5% to −15%: 0.25%) Maximum ripple (peak to peak): 50 mV

Controls: Coarse: "off", "range 1", "range 2". Fine: continuous.

THE '1800' SCALER

Count Capacity: 99,999 counts

Max Count Rate 4,000/sec. (evenly spaced pulses)

Resolution Time: 275μ sec.

Input Sensitivity: 5 volts + ve.

Input Impedance: .01μF into 470 k.

STREAMLINED SERVICING

- Attractively styled cabinets with tilt-up stand can be used singly or stacked.
- Front panels free of all connectors.
- Cabinets slide off without freeing connectors.
- Instantly converted for rack mounting when required.
- Quick replacement plug-in boards for many circuit functions.
- Identical Power Pack in all units simplifies service and replacement.
- All components conservatively rated.
- "Chimney" construction ensures adequate ventilation.
- Every Unit has 100 hours test before despatch.
- More than 90% of Tubes internationally available.
- Fully detailed service Manual.
- Packaged Spares Kits available.

If you are interested in nucleonic counting, but also have to count the pennies and the cubic feet, you should find out more about the I.D.L. '1800 Series'.

Write for details to:

ISOTOPE DEVELOPMENTS LTD

BEENHAM GRANGE, ALDERMASTON, WHARF, Nr. READING: BERKS.

Telephone: Woolhampton 2121. Telegrams: Devisotope Woolhampton.

London Office: 120 Moorgate, London, E.C.2. Telephone: Metropolitan 9641.

THE RADIOCHEMICAL CENTRE

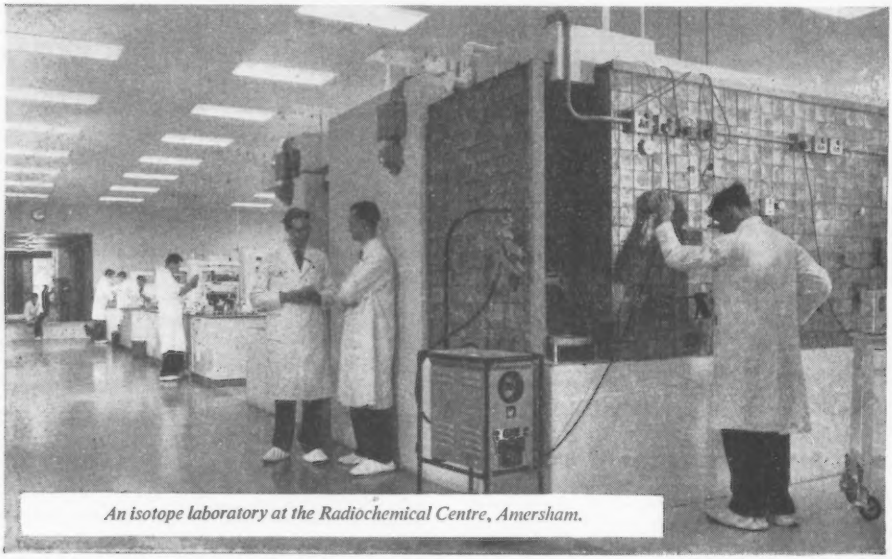
(*United Kingdom Atomic Energy Authority*)

AMERSHAM · BUCKINGHAMSHIRE · ENGLAND

offers you

- An unequalled experience in the preparation of radioactive materials.
- A range of over five hundred isotopically labelled compounds.
- A delivery service controlled by people with a complete knowledge of the exacting requirements and international regulations applying to the distribution of radioactive materials.
- A careful study and execution of your demands.

A comprehensive catalogue is available on request.



An isotope laboratory at the Radiochemical Centre, Amersham.

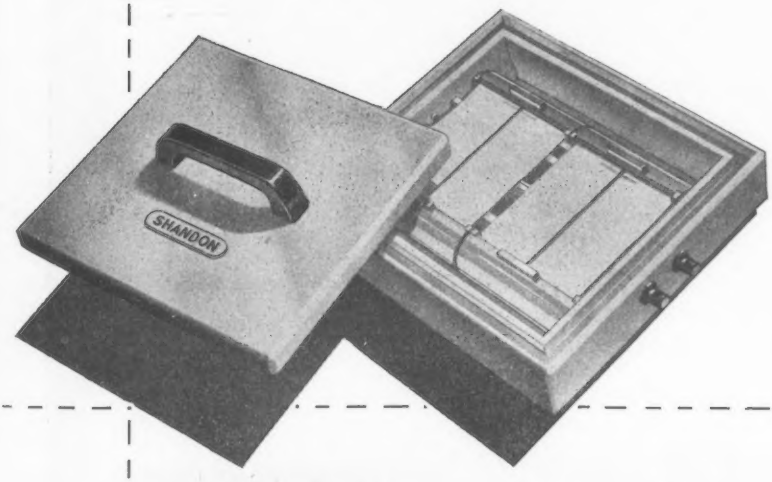
TAS/RC.44

The NEW SHANDON UNIVERSAL **ELECTROPHORESIS APPARATUS** after KOHN

Shandon's latest version of their Universal Electrophoresis tank incorporates important improvements, adding still further to the equipment's versatility, economy and convenience of operation. The bridge gap is now readily adjustable to take four strip lengths, and an improved tensioning method both simplifies the handling of thin membrane and allows a greater economy in its use.

Vapour-tight and draught-proof, the apparatus is ideal for use with cellulose acetate membrane strips. This technique, which will clearly demonstrate several fractions not seen at all on filter paper, gives sharp rapid separation—particularly of albumin. Adsorption is low and there is practically no tailing of fractions. The membrane can be made transparent to both visible and U.V. light. Only a very small sample is necessary.

Write for descriptive leaflet today.



SHANDON **SHANDON SCIENTIFIC COMPANY LTD**

6, CROMWELL PLACE, LONDON, S.W. 7. TELEPHONE KNIghtsbridge 1131

DIFCO

microbiological reagents and media

ANTISERA and ANTIGENS

ENTEROPATHOGENIC COLI

★

KLEBSIELLA

★

SALMONELLA SHIGELLA

★

BRUCELLA LEPTOSPIRA
STREPTOCOCCI

★

C PROTEIN ANTISERUM AND
STANDARD

★

INFECTIOUS MONONUCLEOSIS
ANTIGENS AND STANDARDS

Over 60 years' experience assures

UNIFORMITY • STABILITY • ECONOMY

Difco Laboratories

Prompt delivery from U.K stock. Write for Difco Manual and technical leaflets to the sole agents:

BAIRD & TATLOCK (LONDON) LTD., CHADWELL HEATH, ESSEX, ENGLAND

Branches in London, Manchester and Glasgow *Agents throughout U.K. and all over the world.*

BTL *complete laboratory service*

TAS/DO.5

THE QUÉRE TITROMETER

has wide applications in recording physico-chemical processes

The Quére Titrometer is a precision instrument for recording titrations accurately. Equipped with an automatically variable recording table to suit the gradient of the curve, it permits the rate of advance to be reduced when the rate of change increases. Reagent flow is controlled by the table mechanism and by changing the jet of the syringe, the volume being represented by the abscissa of the graph and the state of mixture by the ordinate. The circle method determines accurately the end points of titration. *The circuits permit all kinds of potentiometric titrations and with special attachments it is possible to perform conductimetric or polarised-electrode titrations.*

* **PHOTOMETRY** The Quére strip photometer attachment is provided with filters and a vacuum type photocell giving accurate and stable readings.
* **USING A LIQUID MEDIUM** photometric attachment to this Recorder allows any photocolourimetric titration, recording photometric variations instead of equivalent points.
* **INTEGRATION** The Recorder automatically integrates the curve as it is traced. The results are entered on the axis of the ordinate of the curve by means of an electric pen marking 'tens' and 'units'.
* **RECORDING TIME FUNCTIONS** The Titrometer is fitted with a zero drift correcting circuit using an automatic potentiometer. To make accurate measurements it is necessary only to check the zero periodically; the instrument itself will then introduce the necessary corrections.

Write for fully illustrated and descriptive leaflet

Tinsley

H. TINSLEY & CO. LTD.
Werndee Hall, South Norwood,
London, S.E.25
Tel: ADDiscombe 6046-8

JSP 5719

SYMPOSIA OF THE BIOCHEMICAL SOCIETY

1.	The Relation of Optical Form to Biological Activity in the Amino-Acid Series.	*5s. net*
2.	The Biochemical Reactions of Chemical Warfare Agents.	*Out of print*
3.	Partition Chromatography.	*Out of print*
4.	Biochemical Aspects of Genetics.	*Out of print*
5.	Biological Oxidation of Aromatic Rings.	*8s. net*
6.	Biochemistry of Fish.	*12s. net*
7.	Biochemistry of Fertilization and the Gametes.	*9s. 6d. net*
8.	Metabolism and Function in Nervous Tissue.	*12s. 6d. net*
9.	Lipid Metabolism.	*13s. net*
10.	Immunochemistry.	*12s. 6d. net*
11.	Biological Transformations of Starch and Cellulose.	*10s. 6d. net*
12.	Chemical Pathology of Animal Pigments.	*12s. 6d. net*
13.	The Biochemistry of Vitamin B_{12}.	*20s. net* *In Paper Covers, 13s. 6d. net*
14.	The Structure of Nucleic Acids and their Role in Protein Synthesis.	*Out of print* *In Paper Covers, Out of print*
15.	Metals and Enzyme Activity.	*21s. net* *In Papers Covers, 15s. net*
16.	The Structure and Function of Subcellular Components.	*22s. 6d. net* *In Paper Covers, 15s. net*
17.	Glutathione.	*22s. 6d. net* *In Paper Covers, 15s. net*

CAMBRIDGE UNIVERSITY PRESS
BENTLEY HOUSE, 200 EUSTON ROAD, LONDON, N.W. 1

THE BIOCHEMICAL JOURNAL

Index of Authors and Subjects

1947–1957 Volumes 41–65

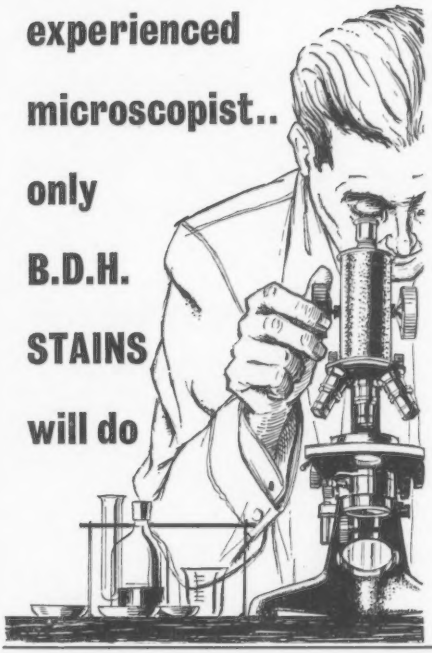
EDITED BY F. CLARK AND F. G. CROSSE

Price 50s. (*30s. to members of the Biochemical Society*)

Orders to

CAMBRIDGE UNIVERSITY PRESS
BENTLEY HOUSE, 200 EUSTON ROAD, LONDON, N.W. 1

For the experienced microscopist.. only B.D.H. STAINS will do



His experience confirms that the standardized and carefully controlled methods employed in the manufacture of B.D.H. Stains ensure that uniform materials are produced which enable him to obtain consistent results, time after time.

A new and completely rewritten edition of the booklet about B.D.H. Standard Stains, entitled 'Biological Stains and Staining Methods', has now been published and may be obtained on request free of charge.

BDH

THE BRITISH DRUG HOUSES LTD.
B.D.H. LABORATORY CHEMICALS DIVISION
POOLE DORSET

ROCHE

Biochemicals for Research

5-HYDROXYINDOLYL-3-ACETIC ACID
(DIETHYLAMMONIUM SALT)

10 mgms. 8/-
100 mgms. 56/-

DL-5-HYDROXYTRYPTOPHAN

25 mgms. 13/6
100 mgms. 40/-

ACETYLTHIOCHOLINE IODIDE

1 gm. 25/-
10 gm. 225/-

BUTYRYLTHIOCHOLINE IODIDE

1 gm. 25/-
10 gm. 225/-

PYRIDOXAL-5'-PHOSPHATE

100 mgms. 18/-
1 gm. 162/-

SERotonin CREATININE SULPHATE

100 mgms. 12/6
1 gm. 112/6

ROCHE PRODUCTS LIMITED, 15 Manchester Square, London, W.1

Telephone: Welbeck 5566

IN A HURRY!

FOR RESEARCH BIOCHEMICALS?

Just phone our EMERGENCY number . . . MOntrose 2-0214, Cleveland, Ohio . . . and your ORDER WILL BE SHIPPED WITHIN ONE HOUR! Even our normal service is fast . . . every order shipped within 8 hours of receipt! And of course, every NBCo biochemical is the highest quality available commercially . . . at the lowest possible price! Yes, you get the BEST for LESS when you buy from NBCo . . . and you get DELIVERY when you NEED it! Why not call us the next time you need ANY biochemicals?

Our stock of more than 2,500 items includes:

- Over 300 Amino Acids
- Over 90 Peptides
- More than 200 Nucleoproteins, Purines, Pyrimidines
- Miscellaneous Biochemicals
- Vitamins
- Enzymes-Crystalline, Purified
- Growth Factors
- Steroid Hormones
- Biological Salt Mixtures
- Biological Test Materials
- Carbohydrates
- Purified Proteins
- Fatty Acids
- Antibiotics
- Alkaloids
- Glandular Substances

OUR NEW OCTOBER 1959 CATALOG
containing more than 2,500 items is now ready. Fill out coupon below and mail today for your free copy.

Name _____

Firm or Organization _____

Address _____

City _____ Zone _____ State _____

NUTRITIONAL BIOCHEMICALS CORPORATION
21019 MILES AVENUE
CLEVELAND 28, OHIO

[*The*

The 385
on Thurs

Introdu
(*Che*

To date,
from nat
well defi
tively lit
They occ
distribute
the Rubi
groups
quinones
quinones
quinones
animals,

Chemi
quinones
quinones
and there
and struc
istic che
common
great sig
may par
and oxid
probably
ing of t
1:4-Addit

Quinone
Scho

Occurre
cated cas
normal c
vitamin
a variety
yield. Al
para-quin
quinone
being hyd
and meth

a

[*The Editors of the Biochemical Journal accept no responsibility for the Reports of the Proceedings of the Society.*]

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **385th Meeting** *of the Society was held in the Department of Biochemistry, University of Liverpool, on Thursday and Friday,* 16 *and* 17 *July* 1959, *when the following papers were read:*

COLLOQUIUM ON 'NATURALLY OCCURRING QUINONES AND THEIR BIOLOGICAL ROLES'

Introduction: Distribution and Nature of the Naturally Occurring Quinones. By R. H. THOMSON. (*Chemistry Department, University of Aberdeen*)

To date, about 170 quinones have been isolated from natural sources. Their chemical structure is well defined in the majority of cases, but comparatively little is known of their biological function. They occur mainly in flowering plants (75 quinones distributed amongst 30 families, a third of them in the Rubiaceae) and fungi (55 quinones); smaller groups have been obtained from insects (15 quinones), sea urchins (10 quinones), lichens (10 quinones), bacteria (5 quinones) and conifers (2 quinones). Ubiquinone has been found in higher animals, including man.

Chemically they can be divided into benzoquinones (40), naphthaquinones (40), anthraquinones (65), and higher polycyclic quinones (25), and there is considerable diversity in the pattern and structure of substituents. Only one characteristic chemical reaction—reversible reduction—is common to all of the natural quinones. This is of great significance in a few cases where quinones may participate in electron transport sequences and oxidative phosphorylation reactions, but is probably not important in the biological functioning of the majority of the natural quinones. 1:4-Addition of quinones and thiols is another typical quinone reaction. By interaction with enzymes in this way quinones could interfere with metabolic processes, but again this is only possible with comparatively few of the natural quinones, as the majority lack the unsubstituted quinonoid position, necessary for 1:4-addition. Quinones which may behave in this way include a group of simple alkylated benzoquinones secreted by insects (presumably a defence mechanism), and a few naphthaquinones (juglone, plumbagin) known to be poisonous to animals and/or plants.

Another group of quinones can be classified as isoprenoid derivatives of the basic types. (Perezone is actually a sesquiterpenoid quinone, and the tanshinones are closely related to abietic acid.) These possess side chains, containing from one to ten C_5 units, and include the biologically important ubiquinones and vitamins K. (Other examples: auroglaucin, pirolatin, lapachol, dunnione.) Probably the majority of naturally occurring quinones are biosynthesized directly from acetate units, isoprene units being introduced separately, via mevalonic lactone, to form side chains, or occasionally, additional aromatic rings. This type of quinone is probably of very wide occurrence.

Quinones in Micro-Organisms: Chemistry and Biosynthesis. By J. H. BIRKINSHAW. (*London School of Hygiene and Tropical Medicine, University of London*)

Occurrence and structure. The only well-authenticated case of quinone formation by bacteria in the normal course of metabolism is the production of vitamin K_2. The fungi, on the other hand, produce a variety of quinones, in a few cases in considerable yield. All these quinones, with one exception, are *para*-quinones. They show substitution in the simple quinone structure, the commonest substituents being hydroxyl groups (which may be methylated) and methyl groups (which may be oxidized to $CH_2{\cdot}OH$ or CO_2H). Much less frequently higher alkyl groups and phenyl groups (which may themselves be substituted) occur as substituents.

Examples of quinones belonging to each of the series benzoquinones, naphthaquinones and anthraquinones are known. In each of these groups there are examples of the C–C linkage of two molecules of the quinones to give diquinones. One fungal phenanthrenequinone is known, this is the sole representative of the *ortho*-quinones so far encountered.

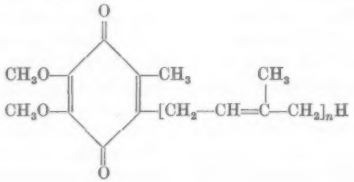
Biosynthesis. Seshadri considered that the orsellinic unit, an invariable component of the lichen acids and depsidones, could give rise to many of the fungal products. He postulated that nuclear oxidation, reduction and methylation might occur. For the benzoquinone derivatives important intermediates are 3- and 5-hydroxyorsellinic acid.

In the case of the anthraquinones a 3:5-dihydroxyphthalic acid unit is combined with a second C_8 unit which has undergone greater modification. The structure of endocrocin and hence of the emodin derivatives follows easily from the condensation of two such C_8 units. Hydroxylated emodin derivatives such as catenarin are obtained as the result of additional nuclear oxidation in the 1-position, considered as taking place in the pre-anthraquinone stages.

The acetate hypothesis of biosynthesis, with which the names of Collie, Robinson and Birch are chiefly associated, claims that many fungal metabolites, and in particular the quinones, are built up from chains of acetate units, combined in some cases with methylation by C_1 units. The hypothesis has been tested by the incorporation in the substrate of acetate labelled with ^{14}C in the carboxyl group. The radioactive quinones thus synthesized were then degraded by suitable methods to determine the labelling of the individual carbon atoms. In three cases examined the labelling was in accordance with the acetate hypothesis.

Ubiquinones. By R. A. MORTON. (*Department of Biochemistry, University of Liverpool, Liverpool* 3)

These compounds have the following structure



They all show a characteristic absorption spectrum with a peak (in *cyclo*hexane) at 272 mμ, $\epsilon_{max.}$ 14 450, inflexion 325 mμ, ϵ_{1120}, and a weak maximum at 407 mμ, $\epsilon_{max.}$ 750. An animal ubiquinone has been studied at Liverpool since 1951 and has been called the 272 mμ substance or SA; it was first found in horse intestinal mucosa and later in rat liver where it occurred in greatly increased amounts in vitamin A deficiency (Lowe, Morton & Harrison, 1953). Baker's yeast was shown to contain a substance at that time indistinguishable from SA (Heaton, Lowe & Morton, 1956). The name ubiquinone was given to SA in 1957 (Morton, Wilson, Lowe & Leat, 1957) because of its wide distribution in animal and other tissues (liver, kidney, heart, blood, pancreas, spleen, brain). It has recently been found in human placenta. It also occurs in fish tissues, e.g. cod liver, heart and muscle were tested. The spadix of the cuckoo-pint (*Arum maculatum*) is an interesting source of an ubiquinone.

Crane, Hatefi, Lester & Widmer (1957) described a quinone (Q 275, mitoquinone or coenzyme Q) isolated from mitochondria) which proved to be the same as SA (ubiquinone). The Wisconsin workers, like the Liverpool group, were handicapped by difficulties experienced in ascertaining molecular weights. The ubiquinone of baker's yeast has a $C_{30}H_{49}$ side chain (Gloor, Isler, Morton, Rüegg & Wiss, 1958), which also occurs in the ubiquinone from *S. cerevisiae* (Lester, Crane & Hatefi, 1958). A $C_{35}H_{57}$ side chain occurs in the ubiquinone from Torula yeast and a $C_{40}H_{65}$ side chain in *A. vinelandii* (Lester *et al.* 1958). Polyisoprenoid side chains also occur in the vitamins K produced by micro-organisms. A hydrocarbon $C_{45}H_{76}$ of similar nature has long been known to occur in liver. The side chain of ubiquinones are formed in the animal and if radioactive mevalonic acid is used it is found that the incorporation into ubiquinone of rat liver is enhanced (Gloor & Wiss, 1959).

REFERENCES

Crane, F. L., Hatefi, Y., Lester, R. L. & Widmer, C. (1957). *Biochim biophys Acta*, **25**, 220.

Gloor, U., Isler, O., Morton, R. A., Rüegg, R. & Wiss, O. (1958). *Helv. chim. Acta*, **41**, 2357.

Gloor, U. & Wiss, O. *Archives Biochem. Biophys.* (1959). **83**, 216.

Heaton, F. W., Lowe, J. S. & Morton, R. A. (1956). *J. chem. Soc.* p. 4094.

Lester, R. L., Crane, F. L. & Hatefi, Y. (1958). *J. Amer. chem. Soc.* **80**, 4751.

Lowe, J. S., Morton, R. A. & Harrison, R. G. (1953). *Nature, Lond.*, **172**, 716.

Morton, R. A., Wilson, G. M., Lowe, J. S. & Leat, W. M. F. (1957). *Chem. & Ind.* p. 1649.

Quinones in Electron Transport and Oxidative Phosphorylation. By E. R. REDFEARN and ALISON M. PUMPHREY*. (*Department of Biochemistry, University of Liverpool, Liverpool* 3)

Quinones possess the property of reversible oxidation-reduction and this suggests that the naturally occurring quinones may function as electron carriers in respiratory enzyme systems. Recently, such a function has been proposed for ubiquinone (coenzyme Q) and for certain members of the vitamin K series.

From work which has been done by groups in Madison and Liverpool it seems that ubiquinone is an active member of the mitochondrial electron transport system. Using mitochondrial preparations from pig heart the Liverpool group has shown that these contain a relatively high concentration of endogenous ubiquinone (4·0 μmoles per g. protein). The endogenous ubiquinone is reduced when succinate or DPNH are added to the preparation under anaerobic conditions and the ubiquinol is reoxidized when the preparation is oxygenated. Antimycin A and BAL had no effect on the reduction reaction but they inhibited the re-oxidation.

In a study of the kinetics of endogenous ubiquinone it was found that the maximal ubiquinone reductase activity obtained was equivalent to 50% of the overall succinic oxidase activity. The ubiquinol oxidase rates were dependent on the buffer used. This is reflected in the steady-state oxidation levels of ubiquinone; in phosphate it was 20–30% whereas in tris it was 70–80%.

* In receipt of a D.S.I.R. research studentship.

The Madison group have shown that the loss of enzymic activity which occurs on extraction of endogenous ubiquinone with organic solvents can be restored by adding ubiquinone as an ethanolic solution to the preparation.

These results suggest that ubiquinone occupies a position on a branch pathway in the respiratory enzyme system and that it is reduced by flavoprotein and oxidized by way of the antimycin A-sensitive region and cytochrome oxidase. A possible function for ubiquinone in oxidative phosphorylation will be discussed.

From results obtained with vitamin K_1-deficient chicks, Martius postulated that vitamin K_1 is an essential cofactor in oxidative phosphorylation. Support for this concept came from experiments in which rat-liver mitochondria or extracts of certain bacteria were irradiated with ultraviolet light. This resulted in a partial depression of the oxidative phosphorylation which could be restored to normal by addition of vitamin K_1. The reactivation is claimed to be due to replacement of the endogenous vitamin destroyed by irradiation. However, the concentrations of vitamin K in mitochondria are apparently much lower than those of known respiratory chain components. This suggest that if vitamin K normally functions in oxidative phosphorylation, then it does so in a form not detectable by the usual methods of assay.

COMMUNICATIONS

Observations on Brain Sphingomyelin Metabolism. By G. B. ANSELL and SHEILA SPANNER. (*Department of Experimental Psychiatry, The Medical School, Birmingham* 15)

It is generally considered that brain sphingomyelin has a low rate of metabolism and is one of the more permanent structures of myelin but, with the exception of some observations by Dawson (1954*a*), few studies have actually been carried out on its biosynthesis *in vivo*. The findings of Rennkamp (1949) and Dawson (1958) that sphingomyelin can be split easily by methanolic HCl to yield sphingosylphosphorylcholine and phosphorylcholine, which can subsequently be separated by paper chromatography, provide a convenient method for determining the uptake of ^{32}P into sphingomyelin *in vivo*.

Brain lipids from the rat were saponified by the method of Schmidt, Benotti, Herschman & Thannhauser (1946) and the alkali-stable fraction hydrolysed with 2N-hydrochloric acid in methanol for 1½ hr. to yield water-soluble phosphate esters and no inorganic phosphorus. When the brains of 5–6 week-old rats were examined it was found that, in addition to the sphingosylphosphorylcholine and phosphorylcholine, at least one other water-soluble ester was present which amounted to about 12% of the total phosphorus. Unidentified water-soluble hydrolysis products of alkali-stable lipids from tissues including brain have also been found by Dawson (personal communication). Approximately 50% of the phosphorus remained chloroform-soluble but subsequent experiments showed that this fraction probably contained unhydrolysed sphingomyelin. On the other hand, the phosphorus of a sample of brain sphingomyelin kindly supplied by Dr J. N. Hawthorne was completely converted to phosphorylcholine and sphingosylphosphorylcholine by the hydrolysis procedure. The presence of phospholipids other than sphingomyelin in the alkali-stable fraction of brain lipids has been previously described by Brante (1949), Dawson (1954*b*) and Carter, Smith & Jones (1958).

The radioactivity of the sphingomyelin phosphorus relative to that of the brain trichloroacetic acid-soluble fraction was used as a measure of ^{32}P incorporation. Measurement of the uptake of ^{32}P into brain sphingomyelin for short periods up to 24 hr. indicated a relatively rapid uptake by young animals in which myelination was actively taking place. For rats 12–15 days old the uptake was approximately 8 μg. P/mg. sphingomyelin P/hr. With increasing age the capacity to incorporate ^{32}P into sphingomyelin decreased and seemed to reach a steady state of 2–3 μg. P/mg. sphingomyelin P/hr. when measurements were made up to 50 weeks of age. These observations are based on the assumption that brain sphingomyelin is homogeneous though there are reasons for believing that this is not so. The findings were discussed in relation to the results of Davison & Dobbing (1958) and Thompson & Ballou (1954) on the relative permanence of brain lipids.

REFERENCES

Brante, G. (1949). *Acta physiol. scand.* **18**, Suppl. 63.

Carter, H. E., Smith, D. B. & Jones, D. N. (1958). *J. biol. Chem.* **232**, 681.

Davison, A. N. & Dobbing, J. (1958). *Lancet*, ii, 1158.

Dawson, R. M. C. (1954*a*). *Biochem. J.* **57**, 237.

Dawson, R. M. C. (1954*b*). *Biochem. J.* **56**, 621.

Dawson, R. M. C. (1958). *Biochem. J.* **68**, 357.

Rennkamp, F. (1949). *Hoppe-Seyl. Z.* **284**, 215.

Schmidt, G., Benotti, J., Herschman, B. & Thannhauser, S. J. (1946). *J. biol. Chem.* **166**, 505.

Thompson, R. C. & Ballou, J. E. (1954). *J. biol. Chem.* **208**, 883.

Ubiquinone and other Unsaponifiable Constituents of the Cod (*Gadus morrhua*). By J. F. Pennock,* R. A. Morton and D. E. M. Lawson.* (*Department of Biochemistry, The University of Liverpool, Liverpool* 3)

Ubiquinones have not hitherto been recorded in fishes. It is now shown that cod contains ubiquinone 50.

Cod hearts and livers obtained from fish landed at Aberdeen were canned and sent from the Torry Research Station to Liverpool together with cod flesh from a similar catch. Cod hearts (250 g.) were digested with ethanolic potash containing pyrogallol and the unsaponifiable extract (0·34 %) was chromatographed on alumina. The results showed the presence of ubiquinone (48·6 μg./g.) heart wet wt., α-tocopherol (*ca.* 12 μg./g.), sterol (*ca.* 2 mg./g.) with small amounts of hydrocarbons, α-tocopherylquinone and 7-dehydro steroid. Cod flesh gave 0·127 % (wet wt.) of unsaponifiable matter containing much sterol, together with detectable amounts of ubiquinone (0·235 μg./g.) and α-tocopherol (3·3 μg./g.).

Cod liver, which had been cooked in the tin was separated into oil and residue and the latter was saponified and the unsaponifiable fraction was studied. The residue was found to contain ubiquinone (2·76 μg./g.), α-tocopherol (24·6 μg./g.), sterol (1·13 mg./g.).

Tinned cod-liver (oil and residues saponified together) gave 0·2 % of unsaponifiable matter from which much sterol was removed by crystallization from light petroleum. The residue was dissolved in methanol and ubiquinone went into the portion insoluble at $-29°$ and was thus separated from vitamins A and E. Chromatography then gave a spectroscopically normal ubiquinone fraction (9·8 μg./g. liver wet wt.).

A larger quantity (1200 g.) of cod hearts was collected at Aberdeen and canned. The aqueous liquor was removed and the residue examined. The unsaponifiable matter (*ca.* 3 g.) was crystallized from light petroleum ($-29°$) and the sterol (2 g.) removed. The remainder on chromatography gave a mixed ubiquinone-tocopherol fraction which was acetylated and re-chromatographed to separate the tocopheryl acetate. A good ubiquinone fraction (40·4 mg. $E^{1\%}_{1\,cm.}$ 272 mμ 164) was purified by crystallization at room temperature from ethanol [m.p. 45·2, $E^{1\%}_{1\,cm.}$ 272 mμ 170, OMe 7·29 %, *M* (calc. from methoxyl value) 855]. This agrees well with the properties of ubiquinone 50 from animal tissues, e.g. pig heart, human kidney. Tocopherol fractions from cod tissue were chromatographed on paper by Dr R. J. Ward and only α-tocopherol was found.

* In receipt of studentships from the Agricultural Research Council and the Department for Scientific and Industrial Research.

The cod tissues were made available from the Torry Research Station, Aberdeen, by courtesy of the Director, Dr G. A. Reay and Dr J. A. Lovern.

The Interconversion of Cholesterol, 7-Dehydrocholesterol and Lathosterol in the Rat. By E. I. MERCER and J. GLOVER. (*Department of Biochemistry, The University of Liverpool, Liverpool* 3)

Previous studies on the absorption of sterols from the intestine of the guinea pig and rat (Glover, Glover & Morton, 1952; Glover & Green, 1957; Glover & Stainer, 1959) indicate that the reduction of the 5–6 bond of 7-dehydrocholesterol to form lathosterol takes place more readily than that of the 7–8 bond yielding cholesterol. In a study on the factors controlling the interconversion of these sterols, the reversibility of the above reductions has been examined in the rat. Cholesterol (50 mg./rat) and lathosterol (27 mg./rat) have been administered separately to groups of two animals and the 7-dehydrocholesterol contents (mg./rat) of their intestinal mucosa estimated 3½–4 hr. after dosing. The values for the lathosterol-dosed groups (0·2–0·7 mg.) were greater than those for controls (0·01–0·08 mg.), whereas the values for the cholesterol groups (< 0·02 mg.) tended to be lower than the control level.

In other experiments 7-dehydrocholesterol, dispersed in aqueous medium (1 mg./ml.), was incubated with inverted intestinal sacs by a similar procedure to that used by Smith, Hauk & Treadwell (1958). The sterol was absorbed and reduced to both lathosterol (18–26 % of sterol added) and cholesterol (8–25 %). In comparable experiments with lathosterol, however, its oxidation to 7-dehydrocholesterol took place only to a small extent (< 1 %). Thus the equilibrium of this *in vitro* system also favours the more saturated sterols. However, it was possible to show some turnover of the sterols by the reductase enzymes on incubating similar preparations with sodium [2-^{14}C]acetate when all three sterols became labelled. The sterols were isolated first as digitonides and then as crystalline azobenzene-4-benzoates, and separated by chromatography. The specific activity of the lathosterol was invariably 3–4 times greater than those of 7-dehydrocholesterol and cholesterol which were similar. These latter results are in agreement with the observations of Brooks & Baumann (1957) on the incorporation of labelled acetate into skin sterols.

It appears that two separate reductase (or dehydrogenase) enzymes are responsible for the interconversion of the above sterols, and that lathosterol comes before cholesterol in the biosynthetic sequence.

We wish to acknowledge with thanks the technical assistance of Mr G. Exell.

REFERENCES

Brooks, S. C. & Baumann, C. A. (1957). *J. biol. Chem.* **229**, 329.
Glover, J. & Green, C. (1957). *Biochem. J.* **67**, 308.
Glover, J. & Stainer, D. W. (1959). *Biochem. J.* **72**, 79.
Glover, M., Glover, J. & Morton, R. A. (1952). *Biochem. J.* **51**, 1.
Smith, A. L., Hauk, K. & Treadwell, C. R. (1958). *Amer. J. Physiol.* **193**, 34.

Hydroxylation of 11-Deoxycorticosterone by Adrenal Glands of Various Mammalian Species. By K. GRIFFITHS and J. K. GRANT. (*Department of Biochemistry, University New Buildings, Teviot Place, Edinburgh*)

The hydroxylation *in vitro* of 11-deoxycorticosterone (DOC) has been studied using homogenates of adrenal glands of various species. The species included animals having the 'non-fatty' type of adrenal glands (ox, sheep, goat and golden hamster) and others (dog, guinea pig, horse, lion, man, mouse, rabbit and rat) having the more common 'fatty' type of gland (Symington, 1959). The glands differ in the fact that the fasciculata and reticularis zones are not distinct in the 'non-fatty' glands in which cells are of the compact reticularis type, whereas the 'fatty' glands have a distinct reticularis and lipid rich fasciculata zone. Incubation conditions and methods of determining steroids were similar to those of Grant (1956).

Rates of 11β-hydroxylation are greater in glands of the 'non-fatty' type. The rat which is a corticosterone secretor, showed an average rate of 11β-hydroxylation considerably lower than that found for animals known to secrete hydrocortisone (Bush, 1953), and in general there was no correlation between rates of 11β-hydroxylation and the known hydrocortisone/corticosterone ratios (Bush, 1953). Our observations thus do not support the view of Hofmann (1957) that the rat differs from most other species examined in failing to produce a 17α-hydroxycorticosteroid (hydrocortisone) on account of the high rate of 11β-hydroxylation in its adrenal glands.

Paper chromatography revealed the presence of a substance X, more polar than corticosterone, among the products resulting from the incubation of DOC with homogenates of 'non-fatty' adrenal glands. Hamster glands form relatively large amounts of X and in addition a further more polar

substance Y. Substance X could not be detected or only appeared in very small amounts among the products obtained with 'fatty' adrenal glands.

X has been tentatively identified with 19-hydroxy DOC on the basis of, chromatographic behaviour before and after acetylation in different solvent systems which permitted the resolution of 18- and 19-hydroxy DOC, formaldehyde formation on alkaline degradation, various colour reactions, identity of spectra of solutions of X and 19-hydroxy DOC in concentrated H_2SO_4, and the occurrence of a hypsochromic shift of $\lambda_{max.}$ from 242 to 239 mμ. on acetylation.

On incubation of 19-hydroxy DOC with a homogenate of hamster adrenals a substance similar to Y was obtained. Change of chromatographic behaviour of Y on acetylation (Zaffaroni & Burton, 1951) suggests that Y may be 11β:19-dihydroxy DOC.

REFERENCES

Bush, I. E. (1953). *J. Endocrin.* **9**, 95.
Grant, J. K. (1956). *Biochem. J.* **64**, 559.
Hofmann, F. G. (1957). *Endocrinology*, **60**, 382.
Symington, T. (1959). *Biochem. Soc. Symposium*, no. 18 (in preparation).
Zaffaroni, A. & Burton, R. B. (1951). *J. biol. Chem.* **193**, 749.

Boundary Electrophoresis of Brain Homogenates Cleared by the Action of Lysolecithin. By W. L. G. Gent. (*Department of Biochemistry, Guy's Hospital Medical School, London, S.E.* 1)

The total clearing of rat-brain homogenate by the action of lysolecithin (Webster, 1957) allows an extension of electrophoretic studies of brain proteins to include components not directly soluble in buffers usual for electrophoretic experiments. Electrophoretic separation of buffer extracts has been reported by several authors (see Robertson, 1957).

Portions of rabbit-brain homogenate (0·125 g. fresh brain per ml. of homogenate in veronal buffer, pH = 8·9, I = 0·075) were incubated for 3 hr. at 37° with various amounts of pure lysolecithin in the range 0–95 mg. lysolecithin/g. of fresh brain, the largest quantity causing complete clarification. The mixtures were dialysed against the buffer at 0°, centrifuged at 10 000 *g* and then subjected to boundary electrophoresis at 1°.

Without addition of lysolecithin the extract resolved into four principal components (A, B, C and D) accounting for *ca.* 30% total protein and having mobilities 3·3, 5·6, 7·5 and $9{\cdot}0 \times 10^{-5}$ cm.^{2}/v sec. respectively. Of these components B accounted for *ca.* 20% total protein, with A, C and D in about equal amounts.

Increasing addition of lysolecithin resulted in an increase of B only, but this increase was accompanied by the separation (in the ascending limb of the cell) of B into several components of not very different mobility, the central portion of the complex showing an increasing mobility to a maximum of $7{\cdot}8 \times 10^{-5}$ cm.^{2}/v sec. when 82 mg. of lysolecithin/g. of fresh brain was used. Since the action of lysolecithin in releasing protein from cellular material is probably connected with its ability to form soluble complexes with cholesterol (Delézenne & Fourneau, 1914) and with phospholipids (Saunders, 1957), it is concluded that the components of the B complex are largely derived from myelinated areas and consequently are of a structural nature. Similar results have been obtained with rat-brain homogenates.

REFERENCES

Delézenne, C. & Fourneau, E. (1914). *Bull. Soc. chim. Fr.*, **14**, 421.
Robertson, D. M. (1957). *J. Neurochem.* **1**, 358.
Saunders, L. (1957). *J. Pharm. Pharmacol.* **9**, 834.
Webster, R. G. (1957). *Nature, Lond.*, **180**, 660.

Ubiquinone in Rats Deficient in Vitamin A. By T. Moore and I. M. Sharman. (*The Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council*)

Lowe, Morton & Harrison (1953) reported that the livers of vitamin A-deficient rats contained fat-soluble material having a band at 275 mμ, with inflexions at 233, 283 and 332 mμ. Previously absorption at the same maximum had been observed by Moore & Rajagopal (1940) in liver extracts from rats given marginal, but not inadequate, intakes of vitamin A. Ward & Moore (1955) confirmed the absorption at 275 mμ in liver extracts from vitamin A-deficient rats, and found similar bands in extracts from rats cured of avitaminosis A, and from a normal pig and guinea pig. By paper chromatography two substances were separated, one with a single maximum at 275 mμ and the other with its main band at 275 mμ and with an inflexion at 330 mμ. Heaton, Lowe & Morton (1955) designated the two substances A and C, and eventually Morton (1958) and his

colleagues identified substance A as a derivative of 5:6-dimethoxy-1-methyl-*p*-benzoquinone, to which the name 'ubiquinone' was given. Heaton, Lowe & Morton (1955) and Cunningham *et al.* (1955) found substances A and C in organs other than liver, in different species and in normal and malnourished animals.

Studies of possible increases in ubiquinone in the livers of vitamin A-deficient rats are difficult because of its instability. Ubiquinone can readily be observed in crude extracts from deficient animals. Before its observation in extracts from normal rats, however, the strongly absorbing vitamin A must be removed, by processes which may cause loss of ubiquinone. Hence the suggested increase in ubiquinone in avitaminosis A might merely reflect its more ready estimation in the absence of the vitamin. We have therefore examined livers from vitamin A-deficient rats, and others from rats kept healthy by doses of 40 i.u. of vitamin A weekly. This allowed the storage of only trivial amounts of vitamin A in the liver. The livers were digested with alcoholic potash and pyrogallol, the insoluble residue was extracted with ether, and the absorption spectra measured in cyclohexane. For eight normal livers ubiquinone (uncorrected) was 266–436 μg./g. (mean 337) as against 340–832 (mean 522) for eight avitaminotic livers. This difference in concentrations was significant ($0{\cdot}01 < P < 0{\cdot}02$). Total amounts of ubiquinone, however, were about the same in the normal and in the smaller avitaminotic livers (means 3740 and 3340 μg.). Loss of liver weight may therefore play a part in raising the concentration. In most spectra there seemed little evidence that substance C contributed substantially to the absorption at 275 mμ. Thus there was seldom evidence of more absorption at 332 mμ than was to be expected from traces of vitamin A. No significant difference was found in the concentration of ubiquinone in hearts (means 485 and 518 μg./g.).

REFERENCES

Cunningham, N. F., Lowe, J. S., Mervyn, L., Morton, R. A. & Vernon, J. (1955). *Biochem. J.* **60**, xviii.

Heaton, F. W., Lowe, J. S. & Morton, R. A. (1955). *Biochem. J.* **60**, xviii.

Lowe, J. S., Morton, R. A. & Harrison, R. G. (1953). *Nature, Lond.*, **172**, 716.

Moore, T. & Rajagopal, K. R. (1940). *Biochem. J.* **34**, 335.

Morton, R. A. (1958). *Nature, Lond.*, **182**, 1764.

Ward, R. J. & Moore, T. (1955). *Biochem. J.* **59**, xv.

Chromatographic Separation on Alumina of the Total Cerebroside plus Sulphatide from Rat Brain.

By C. Long and D. A. Staples. (*Biochemistry Division, Physiology Department, Institute of Basic Medical Sciences, Royal College of Surgeons of England, Lincoln's Inn Fields, London, W.C. 2*)

The chromatographic behaviour of cerebroside has previously been studied on columns of silicic acid (Weiss, 1956; Payne & Platt, 1958) and Florisil (Radin, Brown & Lavin, 1956). In each case, the starting material had undergone some preliminary purification from other lipids by solvent fractionation. Florisil was shown to separate cerebroside from phospholipids but not from cholesterol or triglyceride (Radin *et al.* 1956). On silicic acid, it has been our experience that only about 75% of the cerebroside plus sulphatide can be eluted before the appearance of an aminophospholipid fraction. The aim of the present work has been to attempt to isolate the total cerebroside plus sulphatide from rat-brain lipids, without previous fractionation other than removal of water-soluble contaminants.

Rat brain was homogenized with 19 vol. of $CHCl_3$-methanol (2:1, v/v), and the lipid extract was washed with 0·1 M-KCl (Folch, Lees & Sloane-Stanley, 1957). The washing procedure removed the strandin (Long & Staples, 1959) and other water-soluble material, but the lipids remained in the $CHCl_3$-rich phase. The latter was then applied to an alumina column (13 g.; 17 cm. × 1 cm. diam.) and the column was washed with $CHCl_3$–methanol (50:50, v/v) in order to elute cholesterol, triglyceride and choline-containing phospholipids (Rhodes & Lea, 1957). Gradient elution was then applied. The mixing chamber contained 125 ml. of $CHCl_3$–methanol (50:50, v/v) and the reservoir contained 150 ml. of $CHCl_3$–methanol–water (45:45:10, by vol.). Fractions (25 ml.) were collected and the whole of the cerebroside plus sulphatide was eluted in fractions 3–9. From fraction 10 onwards, ethanolamine-containing phospholipid was eluted. On analysis, the combined cerebroside plus sulphatide fractions were found to be free from cholesterol, phosphorus and amino N. The ratio, galactose:sphingosine N:total N, was 1:1:1, as demanded by theory.

Our thanks are due to Professor D. Shapiro (Weizmann Institute, Rehovoth, Israel) for gifts of synthetic cerebroside and sphingomyelin, which were used to standardize the analytical methods for galactose and sphingosine.

REFERENCES

Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957). *J. biol. Chem.* **226**, 497.
Long, C. & Staples, D. A. (1959). *Biochem. J.* **71**, 24P.
Payne, S. & Platt, B. S. (1958). *Proc. Nutr. Soc.* **17**, xvi.
Radin, N. S., Brown, J. B. & Lavin, F. B. (1956). *J. biol. Chem.* **219**, 997.
Rhodes, D. N. & Lea, C. H. (1957). *Biochem. J.* **65**, 526.
Weiss, B. (1956). *J. biol. Chem.* **223**, 523.

Effects of pH and Temperature on the Reduction of Periodates by Inositol. By G. H. Sloane-Stanley and Pamela A. Lock. (*Biochemistry Division, Physiology Department, Royal College of Surgeons, Lincoln's Inn Fields, London, W.C.* 2)

Inositol has often been assayed by oxidation with HIO_4, usually under conditions giving a molar ratio (R) of periodate reduced to inositol taken of 6·0 (e.g. Platt & Glock, 1943: mM-HIO_4, 7°, 48 hr.).

With 2 mM-HIO_4 at 50° for 1·5 hr., as used by Le Baron, Folch & Rothleder (1957), R has been found to be 5·6 (4·8 after 1·5–3 hr. at 55° with 4 mM-HIO_4); if it was raised by increases of pH, temperature, time or ionic strength, the lower concentrations of inositol usually gave disproportionately high results.

However, at 55° in solutions buffered at pH 5·5 (acetate; phthalate reacted) or 6·5 (phosphate), R rose to 6·5 or 8, i.e. to Fleury's (1951) theoretical maximum, independent of the inositol concentration; but at pH 6·5 and 60° or 70° (R = 10), the lower concentrations of inositol gave disproportionately low results. The apparent discrepancy between these results and Malaprade's (1933; cf. Böhm & Richarz, 1954) will be discussed.

In all these experiments, periodate was estimated iodimetrically at pH 6·5, the iodine being determined either by titration (Platt & Glock, 1943) or spectrophotometrically (Sloane-Stanley, 1959); if the latter, each buffered sample of about 0·5 ml. was treated with 0·1 ml. of 24 % KI, allowed to react, diluted to 3·25 ml. with water (KI solutions caused subsequent gradual increases in extinction), and read at 445 mμ ($E = 0·45/\mu$mole in a 1 cm. cell, $0·525/\mu$mole in a half-inch specimen tube).

REFERENCES

Böhm, P. & Richarz, G. (1954). *Hoppe-Seyl. Z.* **298**, 110.
Fleury, P. (1951). *Bull. Soc. Chim. biol.* **33**, 1061.
LeBaron, F. N., Folch, J. & Rothleder, E. E. (1957). *Fed. Proc.* **16**, 209.
Malaprade, L. (1933). *Bull. Soc. Chim. Fr.*, (5), **1**, 833.
Platt, B. S. & Glock, G. E. (1943). *Biochem. J.* **37**, 709.
Sloane-Stanley, G. H. (1959). Following abstract.

Aspects of Spectrophotometric Iodometry. By G. H. Sloane-Stanley. (*Biochemistry Division, Physiology Department, Royal College of Surgeons, Lincoln's Inn Fields, London, W.C.* 2)

Although the spectrophotometric estimation of iodine in solutions of KI is well understood (e.g. Custer & Natelson, 1949), the method is surprisingly little used for the many analyses ending in iodine determinations, for which it offers great advantages in speed and sensitivity to laboratories having spectrophotometers.

Suitable wavelengths for various concentrations of iodine in 2–4 % KI in approx. 50 % methanol have been found to be about: 290 mμ for 10^{-5}N, 410 mμ for 10^{-4}N, 470 mμ for 10^{-3}N and 580 mμ for 10^{-2}N. For analyses of large numbers of samples, optically matched half-inch flat-bottomed specimen tubes with corks make excellent substitutes for the usual square cells, being very much cheaper and fitting the same holders; before a reading is taken, however, each tube must be centred in the light beam by finding its position of maximum transmission.

Spectrophotometric iodometry has been applied to the estimation of periodate (Sloane-Stanley & Lock, 1959) and also to Siggia & Edsberg's (1948) method for vinyl ethers, although for plasmalogens (cf. Rapport & Franzl, 1957), chloroform:methanol:24 % aqueous KI (3·75:3·75:1) has had to be used, both mixtures being diluted with methanolic KI before reading. Other uses for spectrophotometric iodometry were described while this work was in progress, by Beadle (1958) and by Heaton & Uri (1958).

REFERENCES

Beadle, L. C. (1958). *J. exp. Biol.* **35**, 556.
Custer, J. J. & Natelson, S. (1949). *Anal. Chem.* **21**, 1005.
Heaton, F. W. & Uri, N. (1958). *J. Sci. Fd Agric.* **9**, 781.
Rapport, M. M. & Franzl, R. E. (1957). *J. Neurochem.* **1**, 303.
Siggia, S. & Edsberg, R. L. (1948). *Anal. Chem.* **20**, 762.
Sloane-Stanley, G. H. & Lock, P. A. (1959). Preceding abstract.

A Method for the Specific Differential Estimation of Cholic and Chenodeoxycholic Acids in Human Plasma. By R. H. P. REID and G. S. BOYD. (*Department of Biochemistry, University of Edinburgh*)

A method has been developed for the estimation of these two acids in plasma which involves the chromatographic separation of these acids followed by spectrophotometric analysis by a modified Pettenkoffer reaction. It is admirably suited for estimations on certain pathological plasma samples and can be modified for use on normal plasma. The procedure for plasma from jaundiced subjects is as follows: 2·0 ml. of plasma is extracted twice with 25 ml. ethanol and the ethanolic extracts are pooled and evaporated to dryness. The residue is dissolved in 25 ml. 0·1 M-$NaHCO_3$–Na_2CO_3 buffer and extracted with an equal volume of light petroleum (60–80°). The aqueous phase is transferred to a nickel crucible using 25 ml. 5 N-NaOH in the process, the crucible is covered, and autoclaved at 110° for 3 hr. After cooling, the solution is made acidic to litmus by the addition of concentrated hydrochloric acid and extracted with 3 × 70 ml. chloroform–ethanol (9:1). The extracts are taken to dryness *in vacuo* and the residue transferred to a boiling tube with ethanol. The ethanolic solution is evaporated to dryness and dissolved in 0·75 ml. 70% acetic acid. This solution is absorbed into 0·75 g. celite, slurried in light petroleum (60–80°) and packed on the top of a Celite column (4 g., height 4·5 in.) employing the partition system described by Matschiner *et al.* (1957). The column is then gradient eluted with successively increasing amounts of benzene (6 gradient steps with 20% increments—each step volume of 25 ml.). Cuts of volume 10·0 ml. are taken and evaporated to dryness by heating in an oven for at least 8 hr. at about 70°. The cuts are then analysed by addition of 5·0 ml. of 70% H_2SO_4 followed by 1·0 ml. of 0·25% furfuraldehyde. After 70 min. at room temperature the pink colour is measured at 5100 Å and the concentrations of the two acids calculated by comparison with the appropriate calibration graphs. This sensitive modified Pettenkofer reaction used in conjunction with the rest of this procedure appears to be specific for cholic acid and chenodeoxycholic acids. The colour is represented by a sharp absorption maximum at 5100 Å.

Cholic and chenodeoxycholic acids are completely separated on these columns and the background reading over the entire range of cuts is consistently low. Recovery experiments on 100 μg. sodium taurocholate give recoveries of 75% ± 4%. This is also the efficiency of the procedure when applied to pure solutions.

REFERENCE

Matschiner, J. T., Mahowald, T. A., Elliott, W. H., Doisy, E. A., jun., Hsia, S. L. & Doisy, E. A. (1957). *J. biol. Chem.* **225**, 771.

The Effect of Dietary Linoleic Acid on Serum Cholesterol in the Rat. By G. S. BOYD and E. BARBARA MAWER. (*Department of Biochemistry, University of Edinburgh*)

The feeding of linoleic acid or oils rich in this acid to rats has been shown to lower serum cholesterol below that of rats on a saturated fat diet (Avigan & Steinberg, 1958). An increased rate of synthesis of cholesterol by livers of linoleate-fed rats has been reported by Merrill (1958) and Mukherjee & Alfin-Slater (1958). The present work was undertaken to investigate the effect of dietary linoleate on the serum cholesterol of the rat.

White male Wistar inbred rats were fed a stock low-fat diet: 70% wholemeal flour, 25% dried skimmed milk, 5% dried yeast. After 7 days the animals were divided into three groups: (1) controls on stock diet, (2) 10% calories replaced by methyl linoleate, (3) 10% calories as methyl stearate. After 8 days on the diets each animal was injected i.p. with 30 μc [1-^{14}C]acetate (100 μmoles). Over the next 100 hr. blood samples were withdrawn from the tail and the specific activity of the serum cholesterol determined. From a plot of the log of the specific activity against time, the rate of incorporation of ^{14}C into cholesterol at zero time was calculated for each group and used as an index of the rate of synthesis of serum cholesterol.

The methyl linoleate feeding resulted in the 'half life' of the serum cholesterol being decreased to 60% of the control value and the rate of synthesis increased to 160% of the control. On feeding methyl stearate the converse was observed, the 'half life' being increased and the rate of synthesis decreased. The experiment was repeated using sunflower seed oil (iodine value 110), which gave similar results to linoleate feeding. The serum cholesterol levels of linoleate-fed animals were lower than those of stearate-fed or of the control animals. In all cases the animals grew normally

and showed no signs of essential fatty acid deficiency.

It is suggested that linoleic acid may act as a cofactor in the metabolism of cholesterol in the rat, its presence increasing the rate of turnover of serum cholesterol.

REFERENCES

Avigan, J. & Steinberg, D. (1958). *Proc. Soc. exp. Biol., N.Y.*, **97**, 814.
Merrill, J. M. (1958). *Fed. Proc.* **17**, 111.
Mukherjee, S. & Alfin Slater, R. B. (1958). *Arch. Biochem. Biophys.* **73**, 359.

Preliminary Observations on the Incorporation of [2-^{14}C]Acetate and [2-^{14}C]Mevalonate into the Unsaponifiable Matter of Isolated Chromatophores of *Rhodospirillum rubrum*. By B. H. Davies and T. W. Goodwin. (*Department of Biochemistry, The University of Liverpool, Liverpool* 3)

Spirilloxanthin, the major carotenoid pigment of *Rhodospirillum rubrum*, is found exclusively in the chromatophores. It is accompanied in the unsaponifiable fraction of chromatophores by a highly polar material which is strongly adsorbed on alumina (Land, 1955) and which gives an R_F of 0·72 in reversed phase paper chromatography using solvent 'C' of Kodicek & Ashby (1954). The spirilloxanthin fraction is relatively more strongly labelled than this compound (which gives none of the characteristic sterol tests) when [2-^{14}C]mevalonate is used than when [2-^{14}C]acetate is used.

[2-^{14}C]Acetate is completely metabolized within 24 hr. by growing cells of *R. rubrum*, incubated anaerobically in the light, and 6–7 % of the total activity is found in the unsaponifiable fraction. On the other hand, under the same conditions, [2-^{14}C]mevalonate is insignificantly metabolized, only about 0·10–0·20 % being found in the unsaponifiable fraction.

Chromatophores prepared by ultrasonic disintegration were resuspended in M/15 phosphate buffer (pH 7·0) anaerobically in the light for 4 hr. in the presence of the labelled substrate. [2-^{14}C]-Acetate was incorporated to the extent of about 0·2 %, and [2-^{14}C]mevalonate to about 0·03 %. Thus in isolated chromatophores, incorporation of acetate is reduced to a much greater extent than is mevalonate incorporation. Attempts to increase the uptake of both acetate and mevalonate by isolated chromatophores have been made by adding (*a*) co-factors (e.g. ATP, TPN and Mn^{2+}), (*b*) a metabolizable substrate (e.g. malate), and (*c*) the supernatant fraction from disintegrated cells. No significant improvement has yet been achieved.

The [2-^{14}C]mevalonate was kindly supplied by Hoffmann-La Roche and Co. (Dr O. Isler); B. H. D. is in receipt of an A.R.C. Research Studentship.

REFERENCES

Kodicek, E. & Ashby, D. R. (1954). *Biochem. J.* **57**, xii.
Land, D. G. (1955). Ph.D. Thesis. University of Liverpool.

Non-esterified Fatty Acids in Sheep Plasma. By D. B. Lindsay. (*A.R.C. Institute of Animal Physiology, Babraham, Cambridge*)

In man (see Frederickson & Gordon, 1958) the non-esterified fatty acids (NEFA) found in plasma are mainly of chain length greater than C_{14} and probably arise from adipose tissue. The plasma level rises on fasting overnight, quickly falling after taking a meal or glucose.

In ruminants, the presence of volatile fatty acids in plasma is well known. But as little information is available concerning the long chain NEFA this has been investigated. It has been found that in the sheep little change is observed in plasma NEFA after a 24 hr. fast. If, however, the rumen is first emptied, a fast of 24 hr. regularly produces a rise in plasma NEFA. The level falls within an hour or two of putting fresh contents in the rumen; and almost immediately if glucose or acetate is infused intravenously.

When acetate was infused intravenously, and interference with glucose utilization was induced in some way, the plasma concentrations of NEFA and acetate increased. This was observed either by giving insulin to bring the blood sugar below 20 mg./100 ml., by giving adrenaline, or by producing alloxan diabetes.

The metabolism of NEFA, acetate and glucose would appear to be interrelated: this may be because (*a*) glucose is required for the oxidation of both long- and short-chain fatty acids; or (*b*) in the presence of glucose, acetate is used for fat synthesis in adipose tissue and if fat synthesis is reduced, long-chain fatty acids are released.

Experiments with ^{14}C-labelled acetate are being carried out to distinguish between these possibilities.

REFERENCE

Frederickson, D. S. & Gordon, R. S. (1958). *Physiol. Rev.* **38**, 585.

Amino Acid Activating Systems from Pig Liver. By L. R. FINCH* and PRISCILLA HELE. (*Medical Research Council, Experimental Radiopathology Research Unit, Hammersmith Hospital, London, W.* 12)

The potentialities of pig liver as a source of amino acid activating enzymes have been investigated. During our studies, Acs, Hartmann, Boman & Lipmann (1959) reported the isolation of a threonine-activating enzyme from calf liver.

Finely-chopped (not homogenized) pig liver is stirred with medium (sucrose, 0·35M; $KHCO_3$, 0·035M; KCl, 0·025M) and filtered through cheesecloth. The extract is treated with protamine (about 1 mg./ml. of extract) to allow sedimentation of the bulk of the particles at 1880 *g* and a protein fraction (i) is obtained by precipitation from the supernatant with a mixture of solid $(NH_4)_2SO_4$ (28 g./100 ml.) and potassium acetate (5 g./100 ml.). Activating enzymes for isoleucine and leucine are concentrated in (i); simple studies of this fraction have been made. The material has a very low RNA content and is not precipitated at pH 5·0, unless RNA be added, when precipitation occurs with further purification of the activating enzymes. 'pH 5 enzymes' (ii) prepared from the original extract by the method of Keller & Zamecnik (1956) catalyse activation predominantly of isoleucine and leucine. This is also true of 'pH 5 enzymes' (iii) obtained from rat liver gently disintegrated in a homogenizer.

Activation has been measured by the amino acid dependent [^{32}P]pyrophosphate-ATP exchange, according to Davie, Koningsberger & Lipmann (1956). The average rates (μm-mole of pyrophosphate exchanged/mg. protein/min.) observed for the fractions (i), (ii) and (iii) were 15, 26 and 118 with isoleucine and 13, 20 and 72 with leucine.

Soluble RNA (5-RNA) in the 'pH 5 enzymes' becomes labelled on incubation with [^{14}C]amino acids and ATP as described by Hoagland, Zamecnik & Stephenson (1957). The (average) extent of labelling (μm-moles/mg. S-RNA) of (ii) and (iii) over a 4 min. incubation period was, respectively, 5·4 and 8·7 for isoleucine, 2·8 and 5·3 for leucine, 0·6 and 1·4 for threonine and 0·7 and 0·5 for valine when these [^{14}C]amino acids were present in the incubations at a concentration of 10^{-4}M. No significant amount of label is incorporated into the trace of RNA in (i), but added pig or rat liver S-RNA is readily labelled by this fraction. The labelling depends upon the amount of S-RNA added (cf. Berg & Ofengard, 1958), and upon the source from which it is obtained. Protamine-treated soluble protein from rat liver, prepared according to Sachs (1957) also labels added S-RNA.

* C.S.I.R.O. Overseas Student.

REFERENCES

Acs, G., Hartmann, G., Boman, H. G. & Lipmann, F. (1959). *Fed. Proc.* **18**, 178.

Berg, P. & Ofengard, E. J. (1958). *Proc. nat. Acad. Sci., Wash.*, **44**, 78.

Davie, E. W., Koningsberger, V. V. & Lipmann, F. (1956). *Arch. Biochem. Biophys.* **65**, 21.

Hoagland, M. B., Zamecnik, P. C. & Stephenson, M. L. (1957). *Biochim. biophys. Acta*, **24**, 215.

Keller, E. B. & Zamecnik, P. C. (1956). *J. biol. Chem.* **221**, 45.

Sachs, H. (1957). *J. biol. Chem.* **228**, 1.

A Coloured Reaction Product from (*im*-DNP)histidine and Methoxycarbonyl Chloride. By K. J. CARPENTER (*School of Agriculture, University of Cambridge*) and W. L. JONES and E. L. MASON (*W. S. Kimpton and Sons, Melbourne C.I., Australia*)

A suggested procedure for estimating the 'available lysine' in foods without chromatography involved reaction with fluorodinitrobenzene (FDNB), acid-hydrolysis, extraction with ether, digestion with methoxycarbonyl chloride (MCC) and measurement of the colour that was then extractable into ether (Bruno & Carpenter, 1957). ϵ-DNP-Lysine reacts with MCC without change in colour, to give an ether-soluble derivative (Bailey, 1957).

Hydrolysates prepared in this way from foodstuffs had shown small increases in colour with MCC. These appeared to correspond to an orange band moving more slowly than the lysine derivative on buffered paper (pH 6) with light petroleum 3: *tert.*-amyl alcohol 2, propan-1-ol 1 as the mixed solvent (modification of Blackburn & Lowther, 1951). With insulin, however, almost a doubling of the colour occurred with MCC. Insulin differs from the mixed proteins of most foods in its high histidine:lysine ratio.

The histidine derivative from the hydrolysis of proteins treated with FDNB, believed to be (*im*-DNP)histidine, is colourless (Porter, 1950). However, applying the Bruno & Carpenter (1957) procedure to α-benzoylhistidine methyl ester gave an orange-yellow, ether-soluble colour after

digestion with MCC. Solutions in N-HCl corresponding to 30 μmol./l. of the original amino acids gave the following $E_{1cm.}$ values:

	A. 400 mμ	B. 440 mμ	A/B
Histidine	0·249	0·087	2·85
Lysine	0·274	0·175	1·57

Calculation of the proportions of the two components, in the solutions obtained with several purified proteins (from their extinction ratios at these wavelengths), has given histidine values close to those reported for ordinary analyses.

Colour development is almost entirely inhibited if the diluted acid-hydrolysate is allowed to stand at room temperature with added Na_2S (0·24–0·35 %, w/v) for 5 min. before adjustment to pH 9 and treatment with MCC. No mechanism is suggested for this, nor is it certain that the histidine is present in the hydrolysate as the simple (*im*-DNP)-derivative (cf. Margoliash, 1955). ϵ-DNP-Lysine is apparently unchanged by pre-treatment with Na_2S. *O*-DNP-Tyrosine gives no colour with MCC under our conditions.

We thank Dr K. Bailey, F.R.S. and Dr F. Sanger, F.R.S., for advice and experimental materials.

REFERENCES

Bailey, K. (1957). *Biochem. biophys. Acta*, **24**, 162.
Blackburn, S. & Lowther, A. G. (1951). *Biochem. J.* **48**, 126.
Bruno, D. & Carpenter, K. J. (1957). *Biochem. J.* **67**, 13P.
Margoliash, E. (1955). *Nature, Lond.*, **175**, 293.
Porter, R. R. (1950). *Biochem. J.* **46**, 304.

Some Further Observations on Insulin and Insulin Antagonists in Protein Fractions of Normal Human Serum. By K. W. Taylor, L. Vargas and P. J. Randle. (*Department of Biochemistry, University of Cambridge*)

Earlier observations on the form of insulin in peripheral human blood, using column electrophoresis, have suggested that serum insulin is associated both with the slower moving albumin fractions, and with the β- and γ-globulins (Randle & Taylor, 1958). In the latter fractions, principal insulin activity has been located in a zone migrating on electrophoresis slightly more slowly than the main β-globulin peak (Taylor & Randle, 1959), with lesser activity present in the γ-globulins, due probably to zone spreading on electrophoresis.

Supporting evidence for this work has been sought by using alternative methods of serum protein fractionation. A method chosen in the present study was the alcohol fractionation technique employed by Lever *et al.* (1951). Protein fractions obtained by this method were dialysed against distilled water, freeze-dried, and tested for insulin activity by measuring their effect on the glucose uptake of the isolated rat diaphragm *in vitro* (Randle, 1954). By this means, insulin activity was found largely confined to fractions I and III which consist chiefly of β- mixed with α-globulins. Further confirmation that this effect was due to insulin was obtained by measurements of the effects of these fractions on glycogen deposition in the isolated rat diaphragm. γ-Globulin (fraction II) was inactive when tested for insulin activity, either by glucose uptake, or glycogen deposition.

In contrast to the stimulatory effect observed with fractions I and III a marked inhibition of glucose uptake was obtained with fractions IV, V and VI from some specimens of normal human serum. These fractions consist mainly of albumin, with lesser quantities of α- and β-globulins. This inhibitory material, unlike that described by Vallance-Owen, Dennes & Campbell (1958), lowered glucose uptake below the level of controls. In this respect it resembles the inhibitory substance detected by Baird & Bornstein (1957), in the residues obtained, when insulin is extracted from serum with organic solvents. Preliminary subfractionation of fractions IV, V and VI by column electrophoresis shows that this inhibitory material is not associated with albumin.

REFERENCES

Baird, C. W. & Bornstein, J. (1957). *Lancet*, i, 1111.
Lever, W. F., Gurd, F. R. N., Uroma, E., Brown, R. K., Barnes, B. A., Schmid, K. & Schultz, E. L. (1951). *J. Clin. Invest.* **30**, 99.
Randle, P. J. (1954). *Brit. Med. J.* **1**, 1237.
Randle, P. J. & Taylor, K. W. (1958). *J. Endocrin.* 17, 387.
Taylor, K. W. & Randle, P. J. (1959). *J. Endocrin.* (in the Press).
Vallance-Owen, J., Dennes, E. & Campbell, P. N. (1958). *Lancet*, ii, 336.

Threonine and Riboflavin Biosynthesis in *Eremothecium ashbyii*. By T. W. GOODWIN and A. A. HORTON. (*Department of Biochemistry, The University of Liverpool, Liverpool* 3)

Goodwin & Pendlington (1954) showed that only two amino acids, serine and threonine, specifically stimulated flavino-genesis in the mould *Eremothecium ashbyii*. Experiments with [^{14}C]serine showed that it was incorporated into riboflavin after being converted into a C_1 unit and glycine; C-1 and C-2 of serine were found in C-4*a* and C-9*a* of riboflavin and C-3 of serine in C-2 of riboflavin (Goodwin & Jones, 1956). The mechanism of threonine stimulation has now been studied.

Threonine aldolase, which splits threonine to glycine and acetaldehyde, was detected in homogenates of *E. ashbyii* using the method of Karasek & Greenberg (1957), in which the liberated acetaldehyde is trapped in semicarbazide in the centre well of a Conway unit and the resulting semicarbazone determined spectrophotometrically. As there was always a high endogenous acetaldehyde production the formation of acetaldehyde from added threonine was confirmed by using generally labelled [^{14}C]threonine as substrate, adding carrier acetaldehyde to the centre well after the reaction was complete, and then isolating and recrystallizing the semicarbazone. The product was highly radioactive.

Addition of generally labelled [^{14}C]threonine to growing cultures of *E. ashbyii* yielded labelled riboflavin. There was no significant activity in C-2, C-4, C-5, C-6, C-7, C-8, C-8*a*, C-10*a*, in the methyl groups attached to C-6 and C-7 or in the ribityl side chain. All the activity is thus located in C-4*a* + C-9*a*, that is, in the carbon atoms contributed by glycine (Plaut, 1954); thus threonine is flavinogenic by virtue of its breakdown to 'glycine', which must be the limiting factor in riboflavin synthesis under our cultural conditions.

The failure of glycine itself to stimulate flavinogenesis in *E. ashbyii* suggests that serine and threonine are active because the appropriate aldolase liberates 'active glycine' which can be utilized by the organism which presumably lacks the ability to activate glycine. Indeed, Dulaney & Grutter (1950) reported that *E. ashbyii* cannot use glycine for growth.

The absence of activity in the aromatic ring of riboflavin, even after considerable amounts of [^{14}C]threonine were added to the cultures, indicates that acetaldehyde is not an intermediate in the conversion of pyruvate into the C_4 precursor of this ring (Goodwin & Treble, 1958).

This investigation was supported by the Agricultural Research Council.

REFERENCES

Dulaney, E. L. & Grutter, F. H. (1950). *Mycologia*, **42**, 717.
Goodwin, T. W. & Jones, O. T. G. (1956). *Biochem. J.* **64**, 9.
Goodwin, T. W. & Pendlington, S. (1954). *Biochem. J.* **57**, 631.
Goodwin, T. W. & Treble, D. H. (1958). *Biochem. J.* **70**, 14P.
Karasek, M. A. & Greenberg, D. M. (1957). *J. biol. Chem.* **227**, 191.
Plaut, G. W. E. (1954). *J. biol. Chem.* **208**, 513.

Thyroxine and Visual Pigment Conversion in Larvae of *Rana catesbeiana*. By F. H. WILT (introduced by T. W. GOODWIN). (*Department of Biochemistry, The University of Liverpool, Liverpool* 3)

Wald (1946) originally reported that larvae of the American bullfrog, *Rana catesbeiana*, possessed the photopigment system porphyropsin, which has as its prosthetic group retinene$_2$. However, the adult of this species has rhodopsin in its outer rod segments, the prosthetic group of which is retinene$_1$. Furthermore, Wald indicated that metamorphosing larvae probably contained mixtures of these two visual pigments. A recent investigation of this phenomenon has confirmed and extended these original findings (Wilt, 1959). The essential change occurring during metamorphosis is a replacement of retinene$_2$ and vitamin A_2 in the retina and pigmented epithelium by retinene$_1$ and vitamin A_1, a change involving the reduction of a single carbon-carbon double bond in the β-ionone ring of vitamin A_2. Administration of thyroxine to premetamorphic larvae stimulates premature photopigment conversion. This is a report of attempts to determine the tissue in which thyroxine, or its physiological derivative, exerts its primary effect.

Small pellets of 20 % thyroxine–80 % cholesterol containing 25–50 μg. of thyroxine were either implanted into one eye or into the abdominal cavity of the larvae. Cholesterol is an inert vehicle in this system which results in less widespread diffusion of the hormone to surrounding tissues and into the circulatory system (Kaltenbach, 1953). After 5–9 days of exposure to hormone the eyes of the different experimental groups were collected, pooled, bleached, and extracted; the vitamin A_1 and A_2 contents were quantitatively determined. Thirteen experiments involving 360 larvae were carried out. The relative amounts of vitamins A_1

and A_2 were determined in (*a*) eyes which had contained thyroxine, (*b*) contralateral sham-operated eyes, (*c*) non-operated control eyes, and (*d*) eyes from animals in which the thyroxine had been placed in the abdominal cavity. The results revealed a slight, but reproducible and significant, increase in the relative amount of vitamin A_1 in eyes which had been directly exposed to the hormone. This supports the hypothesis that the rate-limiting thyroxine-responsive step in visual pigment conversion is in the tissues of the eye. Photopigment conversion may well be a favourable system in which to analyse cellular differentiation at the molecular level.

REFERENCES

Kaltenbach, J. C. (1953). *J. exp. Zool.* **122**, 21–40.
Wald, G. (1946). *Harvey Lectures*, **42**, 148–152.
Wilt, F. H. (1959). *Develop. Biol.* (in the Press).

Phosphorylation of Shikimic Acid by Ultrasonic Extracts of Micro-Organisms. By J. A. Fewster. (*Microbiology Unit, Department of Biochemistry, University of Oxford*)

Davis and his colleagues have firmly established the rôle of shikimic acid as a common precursor of the essential aromatic metabolites tyrosine, phenylalanine, tryptophan, *p*-aminobenzoic acid, and *p*-hydroxybenzoic acid in some micro-organisms. Extracts of *Escherichia coli* mutants have been shown to convert shikimic acid 5-phosphate, accumulated by some multiple aromatic mutants which do not grow with shikimic acid, into compound Z_1 (probably the 5-enolpyruvate of shikimic acid) in the presence of phosphoenolpyruvate (Kalan, 1956) and into anthranilic acid in the presence of L-glutamine (Srinivasan, 1959).

Transphosphorylation from ATP to shikimic acid by ultrasonic extracts of micro-organisms has now been studied. Routinely, the phosphorylation has been measured by following disappearance of free shikimic acid, estimated by the method of Gaitonde & Gordon (1958) after deproteinization and precipitation of the phosphate ester by the addition of barium hydroxide and zinc sulphate (Somogyi, 1945). With extracts of *E. coli* 518 decrease in shikimic acid concentration is accompanied by an equivalent decrease in ATP (measured as labile phosphate by the method of Berenblum & Chain, 1938) and the acid-stable product is chromatographically identical with shikimic acid 5-phosphate isolated from the growth medium of *Aerobacter aerogenes* A170–40 (Weiss & Mingioli, 1956). Crude extracts catalyse the disappearance of shikimic acid at a rate of 140 μm-moles/mg. protein/hr. at the pH optimum of 6·5; after centrifugation of the extract at 100 000 ***g*** all the activity (250–300 μm-moles/mg. protein/hr.) is retained in the supernatant fraction. Following dialysis against ethylenediaminetetra-acetic acid a requirement for Mg^{2+} or Mn^{2+} has been demonstrated. The enzyme is not inhibited by 2×10^{-4}M-phenylmercury acetate.

With a multiple aromatic mutant of *E. coli* which responds completely for growth to shikimic acid enzyme formation is not suppressed by growth on an excess of the complete aromatic supplement; enzyme activity is not inhibited by any of the aromatic compounds at a concentration of 10^{-3}M.

The following are the activities (μm-moles/mg. protein/hr.) of crude extracts of some organisms capable of synthesizing for themselves aromatic amino acids: *Acetobacter suboxydans*, 80; *Aerobacter aerogenes*, 130–200; *Arthrobacter globiformis*, 130; *Corynebacterium erythrogenes*, 160; *Pseudomonas ovalis* Chester, 60.

REFERENCES

Berenblum, I. & Chain, E. (1938). *Biochem. J.* **32**, 295.
Gaitonde, M. K. & Gordon, M. W. (1958). *J. biol. Chem.* **230**, 1043.
Kalan, E. B. (1956). Quoted by Weiss & Mingioli (1956).
Somogyi, M. (1945). *J. biol. Chem.* **160**, 69.
Srinivasan, P. R. (1959). *J. Amer. chem. Soc.* **81**, 1772.
Weiss, U. & Mingioli, E. S. (1956). *J. Amer. chem. Soc.* **78**, 2894.

Soluble Enzymes Catalysing the Oxidation of Reduced Diphosphopyridinenucleotide by Molecular Oxygen and Hydrogen Peroxide Isolated from *Bacillus subtilis*. By J. W. Lightbown and M. Kogut. (*National Institute for Medical Research, Mill Hill, London, N.W.* 7)

The presence of enzyme systems transferring electrons from DPNH to molecular oxygen in organisms devoid of cytochrome is well established. The presence of similar systems not involving cytochromes, in organisms which possess cytochromes, has been inferred by several workers. Such inferences have been based on the extent of the cyanide or carbon monoxide-resistant aerobic

respiration (Gary & Bard, 1952) on aerobic growth and respiration in presence of alkyl-quinoline-*n*-oxides which are potent inhibitors of cytochrome electron-transport systems (Lightbown & Jackson, 1956) and on the decrease in cytochrome levels of some aerobic organisms growing at high aeration levels (Moss, 1956; Rosenberger & Kogut, 1958).

Two flavoprotein enzymes catalysing the oxidation of DPNH by molecular oxygen and hydrogen peroxide have been isolated from *Streptococcus faecalis* and described by Dolin (1955). The presence of similar soluble enzymes in cell-free preparations of *Bacillus subtilis* has been reported by Lightbown (1957). Because the presence of cytochromes in crude enzyme extracts cannot be ruled out completely, and because the presence of catalase in these extracts made a quantitative assessment of the enzymes impossible, further purification was undertaken. Three DPNH-oxidizing enzymes were obtained, one of which will be described in the following communication. The other two, a DPNH-oxidase and a DPNH-peroxidase, could not be separated from one another but could be assayed separately. Purification involved initially, lysis of cells with lysozyme and treatment with deoxyribonuclease. Precipitation of nucleic acids and other impurities at pH 5·0. Further fractionation by precipitation between 30 and 70% saturated ammonium sulphate, stepwise acid precipitation between pH 5·0 and 4·5 to remove catalase, followed by treatment with calcium phosphate gel and chromatography on a DEAE anion-exchange column led to about 300-fold purification.

When purified, the oxidase required FMN, and was inhibited by phosphate. Manganese relieved this inhibition. Mg^{2+} and Fe^{2+} had no effect. The peroxidase required FAD for maximal activity. Both enzymes were unstable, but to differing degrees and were further distinguishable by inhibition studies.

Minimum specific activities, as moles DPNH oxidized/min./mg. protein could be calculated. From these and a comparison of DPNH oxidation by crude extracts in the presence of cytochrome-electron-transport inhibitors, the physiological importance of these enzymes may be estimated.

REFERENCES

Dolin, M. I. (1955). *Arch. Biochem.* **55**, 415.

Gary, N. D. & Bard, R. C. (1952). *Bact. Proc.* p. 147.

Lightbown, J. W. (1957). *Giorn. Ital. Chemioter.* **4**, 22.

Lightbown, J. W. & Jackson, F. L. (1956). *Biochem. J.* **63**, 130.

Moss, F. (1956). *Aust. J. exp. Biol. med. Sci.* **34**, 395.

Rosenberger, R. F. & Kogut, M. (1958). *J. gen. Microbiol* **19**, 228.

Isolation and Purification of a Soluble Diaphorase-like Enzyme from *Bacillus subtilis*. By M. Kogut and J. W. Lightbown. (*National Institute for Medical Research, Mill Hill, London, N.W.* 7)

During a study of soluble DPNH-oxidizing enzymes in *Bacillus subtilis* reported in the preceding communication (Lightbown & Kogut, 1959) we were able to isolate and extensively purify a diaphorase-like enzyme which catalyses the transfer of electrons from DPNH to various acceptors, but not to molecular oxygen or hydrogen peroxide. It was found that this enzyme would readily reduce 2:6-dichlorophenolindolphenol in the presence of DPNH. By following the reduction of the dye spectrophotometrically at 625 mμ, the enzyme could be assayed in the presence of the other two enzymes and under aerobic conditions, provided DPNH was in excess.

The purification procedure was that described in the preceding communication and separation of the 'diaphorase' was achieved on the DEAE anion-exchange column where it migrated behind the other two enzymes in acetate buffer pH 4·6 and ionic strength of $\mu = 0{\cdot}1$. Approximately 300-fold purification was achieved in the procedures subsequent to removal of nucleic acids at pH 5·0.

The purified enzyme was rather stable and, using the above assay system, some of its characteristics were investigated. It had a pH optimum in the region of 7·6–8·0 and there was a marked stimulation of activity by increasing the concentration of phosphate. Versene or a mixture of metals had no effect on the enzyme activity. Neither FMN nor FAD were found to be stimulatory. An absorption spectrum of the purified enzyme preparation showed only a very slight shoulder at about 450 mμ.

The activity measured at pH 7·8 in 0·13 M-phosphate buffer and with excess DPNH was proportional to the amount of enzyme added. The Michaelis constant for DPNH determined under these conditions was found to be about $1{\cdot}3 \times 10^{-4}$ M.

The maximum specific activity of the enzyme calculated as moles DPNH oxidized/min./mg. protein was about ten times higher than that of the purified preparation of the other two DPNH-oxidizing enzymes.

Massey (1958, 1959) has shown that pig-heart diaphorase functions as a lipoic dehydrogenase and is concerned in α-keto acid oxidations. Investigations on the activities of our enzyme with different electron donors and acceptors, and its relationship to lipoic acid metabolism are in progress.

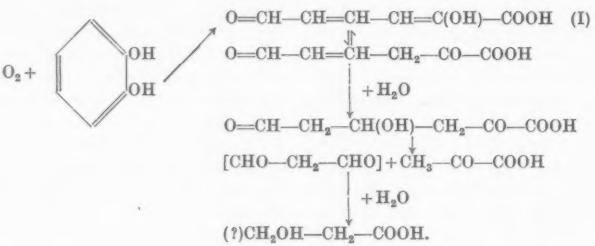
REFERENCES

Lightbown, J. W. & Kogut, M. (1959). *Biochem. J.* (in the Press).
Massey, V. (1958). *Biochim. biophys. acta*, **30**, 205.
Massey, V. (1959). *Biochem. J.* **72**, 7P.

A New Mode of Fission of the Benzene Nucleus by Bacteria. By S. Dagley and D. A. Stopher.
(*Department of Biochemistry, University of Leeds*)

Evans and Stanier and their colleagues have shown that *cis-cis*-muconic acid is the first product of aromatic ring fission when catechol is converted to β-oxoadipate by certain bacteria. We have studied a Gram-negative organism kindly made available by Dr Eileen Pankhurst of the research laboratories at Fulham gasworks and find reactions for aromatic ring fission entirely different from those established for other micro-organisms including the vibrio used by Kilby (1948) when β-oxoadipate was first isolated. However, the present organism is not unique; other strains we have isolated from soil appear to contain this system; and in particular it seems that the pseudomonad studied by Dagley & Patel (1957) may decompose protocatechuic acid by similar reactions. Cells grown with forced aeration in mineral salts media containing *m*-cresol as source of carbon were crushed in the bacterial press of Hughes (1951); the clear extract was then precipitated with ammonium sulphate (60% saturation) and dialysed against water. For each 1 mole of catechol, oxidized rapidly in the presence of extracts, 1 mole of O_2 was consumed, no CO_2 was evolved, no β-oxoadipate was formed and about 1 mole of pyruvate appeared. During the course of the reaction, *cis-cis*-muconic acid when added was not attacked but a compound was produced from catechol which formed an acidic 2:4-dinitrophenylhydrazone. The kinetics of formation and disappearance of this compound can be followed by spectrophotometry and are those expected for an intermediate in the reaction sequence between catechol and pyruvate. The spectrum of this intermediate, which we suggest is compound I in the scheme below, shows a pronounced peak at about 370 mμ at pH 8, largely abolished at pH 3. It is similar to but not identical with the compound reported by Wiss (1956) as the initial product of ring fission of 3-hydroxyanthranilic acid by an enzyme from liver.

Ring fission in this organism does not occur by reactions previously described in bacteria; but further study is needed of the following scheme which we put forward tentatively to account for our present observations:



REFERENCES

Dagley, S. & Patel, M. D. (1957). *Biochem. J.* **66**, 227.
Hughes, D. E. (1951). *Brit. J. exp. Path.* **32**, 97.
Kilby, B. A. (1948). *Biochem. J.* **43**, v.
Wiss, O. (1956). *Z. physiol. Chemie*, **304**, 221.

The Effect of Alcohols on Enzyme Synthesis in *Bacillus cereus*. By D. J. KUSHNER. (*National Institute for Medical Research, Mill Hill, London, N.W. 7*)

Alcohols have previously been found to inhibit lecithinase formation in growing *Bacillus cereus* (Kushner, 1957) and in *Clostridium perfringens* (Jayko & Lichstein, 1958). Their differential effect on enzyme formation and growth seemed of interest for studies on enzyme synthesis in general, and the specificity of the effect has been further investigated.

Growth in the presence of alcohols led to a lowered lipase activity per mg. bacteria (measured manometrically as hydrolysis of tributyrin) in washed intact cells and in cell-free extracts of *B. cereus* strain 569. Ethanol and *n*-propanol had the greatest effect, causing up to 80% reduction in activity. Methanol and *n*-butanol were less effective on a molar basis; the highest concentrations of the last alcohol which still permitted bacterial growth caused up to 50% reduction in lipase activity per mg. bacteria.

All alcohols were without effect on the stability of formed lipase and did not act by selecting a sub-line of more actively lipase-synthesizing cells. Their action appears to be, therefore, on the production of lipase activity.

These alcohols did not inhibit the induced synthesis of penicillinase by this strain nor the constitutive formation of penicillinase by strain 569/H. Growth in the presence of *n*-propanol increased the intracellular phosphatase activity of the culture, whereas the other alcohols had no effect on phosphatase formation.

The possible significance of the fact that, so far, alcohols have been found to inhibit only the synthesis of enzymes acting on fatty substances, will be discussed.

REFERENCES

Jayko, L. G. & Lichstein, H. C. (1958). *Proc. Soc. exp. Biol., N.Y.*, **98**, 63.
Kushner, D. J. (1957). *Nature, Lond.*, **179**, 781.

Pyruvic and Formic Acid Metabolism in *Zymosarcina ventriculi*. By T. BAUCHOP and E. A. DAWES. (*Department of Biochemistry, The University, Glasgow*)

Zymosarcina ventriculi is an anaerobic micro-organism capable of growing under conditions of extremely low pH. It has been little investigated, presumably due to difficulties of culturing. Early fermentation studies with growing cultures (Smit, 1930; Kluyver, 1931) revealed that ethanol and CO_2 were the major products, approximately 1·8 moles of each being produced per mole of glucose. Although small amounts of acetate and H_2 were also found, Kluyver concluded that these results substantiated a yeast-type fermentation.

More recently Milhaud, Aubert & Van Niel (1956) carried out experiments with variously labelled glucose using washed suspensions. They concluded that the labelling found in the fermentation products could be accounted for by Embden–Meyerhof glycolysis. Their results, however, differed markedly from those of earlier workers in that, per mole of glucose, the recoveries of ethanol, acetate, H_2 and CO_2 were 0·8, 0·9, 1·7 and 1·9 moles respectively.

In a general study of the biochemistry of *Z. ventriculi* we have investigated pyruvate and formate metabolism. Intact cells are unable to utilize pyruvate although they actively produce H_2 and CO_2 from formate. Treatment with toluene permitted pyruvate to be utilized but no H_2 was produced, although a toluene-treated preparation has been obtained which yielded H_2 from formate.

A similar preparation fermented pyruvate to yield (moles/mole) 0·66 acetaldehyde, 0·04 acetylmethylcarbinol, 0·12 acetate, 0·14 lactate and 0·85 CO_2. With KOH present in the centre-well of the Warburg vessel aldehyde resin was formed on the filter paper and the recovery of acetaldehyde and other products was reduced, which suggests that acetaldehyde is the precursor of the other products. CO_2 evolution was inhibited completely by *p*-chloromercuribenzoate (0·36 mM) and 52% by copper sulphate (0·36 mM). This evidence indicates the presence of a yeast-type carboxylase.

Approximately equimolar amounts of H_2 and CO_2 were produced from formate by washed suspensions. The formate system is sensitive to atmospheric oxygen and is completely inhibited by cyanide (0·046 mM). Hydrogenase and formic dehydrogenase are also present and it would appear that the organism possesses a typical formic hydrogenylase.

A system which yields formate from pyruvate has not so far been detected although the presence of such a mechanism must be inferred. *Z. ventriculi* thus appears to be the first example of an organism possessing such a system together with a carboxylase.

REFERENCES

Kluyver, A. J. (1931). *The Chemical Activities of Micro-organisms*, p. 39. University of London Press.
Milhaud, G., Aubert, J.-P. & Van Niel, C. B. (1956). *Ann. Inst. Pasteur*, **91**, 363.
Smit, J. (1930). *Pflanzforschung*, 14, *Die Garungssarcinen.*

Some Properties of Adenosine Triphosphatase of Rat-Brain Mitochondria. By J. R. LAGNADO, R. BALÁZS and D. RICHTER. (*M.R.C. Neuropsychiatric Research Unit, Whitchurch Hospital, Cardiff*)

The ATP-ase activity of mitochondrial preparations obtained from Potter–Elvehjem homogenates of rat brain (Balázs & Lagnado, 1959) was studied in 0·06M-tris buffer, pH 7·4. The liberation of inorganic phosphate (IP) from ATP at 25° was proportional, within limits, to tissue concentration and to time. ATP-ase activity was stimulated by magnesium ions (apparent K_a for Mg, 0·2–0·4 mM) as it was by the addition of calcium ions. In the presence of optimal concentrations of Mg (*ca.* 0·003M), the apparent K_a values for ATP ranged from 0·001 to 0·0025M.

Ethylenediaminetetra-acetic acid (EDTA) inhibited ATP-ase activity by competing for Mg ions. The inhibition was diminished by increasing concentrations of ATP (apparent K_I for EDTA in the absence of added Mg and in presence of 0·004M-ATP, 0·0003M). The inhibition of ATP-ase by fluoride was of non-competitive nature with respect to Mg or ATP concentration, and was enhanced by raising the Mg concentration (apparent K_I for NaF were 0·005 and 0·045M in the presence and in the absence of 0·0077M-$MgSO_4$, respectively).

'Ageing' the mitochondria in sucrose (10–40 min. at 37°) decreased ATP-ase activity when assayed in the absence of Mg. Freezing and thawing had no effect.

Brain mitochondrial ATP-ase, unlike that of heart or liver mitochondria (Potter & Recknagel, 1951; Holton, Hülsmann, Myers & Slater, 1957), was relatively insensitive to 2:4-dinitrophenol (DNP) at all concentrations tested (0·00005–0·05M) between pH 5·5 and 9·5. In a typical experiment, the ATP-ase activity (μmoles IP/10 min./100 mg. equivalent fresh tissue) of rat liver (0·29) and brain mitochondria (0·50) was stimulated to 1·33 and 0·62, respectively, by 0·0005M-DNP at pH 7·4. KCl (0·005–0·01M), Mg (0·0005–0·008M), EDTA (0·0001–0·005M) and various combinations thereof did not modify the response of the brain enzyme to DNP.

The activity of brain mitochondrial ATP-ase exhibited three pH maxima in the presence or absence of added Mg (at pH 5·9–6·1, 7·1–7·2 and 8·3–8·5).

Thus, it appears that the brain mitochondrial ATP-ase, like that of bone-marrow, spleen and gut (Maxwell & Nickel, 1954), differs markedly from that of liver or heart mitochondria with respect to DNP, ageing and freezing and thawing; but the brain enzyme behaves similarly to the liver enzyme with respect to pH (Myers & Slater, 1957).

REFERENCES

Balázs, R. & Lagnado, J. R. (1959). *J. Neurochem.* (in the Press).
Holton, F. A., Hülsmann, W. C., Myers, D. K. & Slater, E. C. (1957). *Biochem. J.* **67**, 579.
Maxwell, R. E. & Nickel, V. S. (1954). *Proc. Soc. exp. Biol., N.Y.*, **86**, 846.
Myers, D. K. & Slater, E. C. (1957). *Biochem. J.* **67**, 558.
Potter, V. R. & Recknagel, R. O. (1951). In *Phosphorus Metabolism*, Vol. 1, p. 377. Ed. by McElroy, W. D. & Glass, B. Baltimore: Johns Hopkins Press.

The Destruction of Crocin by Isolated Chloroplasts. By J. FRIEND and A. M. MAYER. (*Low Temperature Research Station, Downing Street, Cambridge*)

The enzymic destruction of β-carotene in plant tissues has been shown to occur mainly in those tissues which contain chlorophyll (Booth, 1958, 1959); it has also been demonstrated in isolated chloroplasts of both spinach beet and sugar beet leaves (Friend & Nakayama, 1959).

We have examined the activity of chloroplasts, prepared from sugar beet leaves, towards the water-soluble carotenoid crocin by incubating chloroplasts with the substrate at 30° in the dark.

The destruction is apparently an aerobic oxidation catalysed by a metallo-protein since it is inhibited by boiling, anaerobiosis, and by diethyldithiocarbamate and KCN, the latter showing 28% inhibition at 10^{-5}M. Other compounds which inhibit the destruction are ascorbic acid, hydroquinone and *p*-benzoquinone at 10^{-3}M and diphenylamine at $1{\cdot}5 \times 10^{-5}$M. Phenazine methosulphate and *o*-phenanthroline at 5×10^{-4}M also inhibit. Zinc acetate (5×10^{-4}M) has little effect but when added with equimolar amounts of *o*-phenanthroline there is an increase in crocin destruction of about 2½-fold; this effect has also been shown for the destruction of endogenous β-carotene in spinach beet chloroplasts (Friend & Nakayama, 1959).

A partial purification of the enzyme has been achieved by treatment of the chloroplasts with sodium deoxycholate in phosphate buffer followed by fractional precipitation with ammonium sulphate.

REFERENCES

Booth, V. H. (1958). *Qualitas Plantarum*, 3/4, 317.
Booth, V. H. (1959). *J. Sci. Fd Agric.* (in the Press).
Friend, J. & Nakayama, T. O. M. (1959). *Nature, Lond.*, (in the Press).

The Ester Sulphate Pattern of Rat Liver. By B. SPENCER. (*Department of Biochemistry, University of Wales, Cardiff*)

The particle-free high-speed supernatant of an isotonic KCl homogenate of rat liver can synthesize 3′-phosphoadenosine-5′-phosphosulphate (PAPS) in the presence of ATP, SO_4^{2-} and Mg^{2+} (cf. Robbins & Lipmann, 1957). At pH 7·3 and 38° and with the concentrations of reactants used in the present experiments, the PAPS concentration in the above system increased at a diminishing rate to a maximum after 75 min. and thereafter the level decreased. Although some PAPS is undoubtedly destroyed by a specific 5′-nucleotidase (Brunngraber, 1958) it was seen, using $^{35}SO_4^{2-}$, that much of the apparent decrease in the rate of PAP^{35}S synthesis was due to the transfer of $^{35}SO_4^{2-}$ from PAP^{35}S to endogenous sulphate-acceptors in the liver preparation.

Chromatography of the incubated liver preparations using either acetone:water (9:1, v/v) or isobutyric acid:0·5N-NH_4OH (5:3, v/v) followed by radioautography showed the presence of $^{35}SO_4^{2-}$, PAP^{35}S and adenosine 3′-phospho[^{35}S]sulphate plus up to 15 other radioactive spots. The pattern did not alter significantly with various liver preparations although some slight differences were observed between those from male and female rats. All the unknown spots appeared to be sulphate esters since, (1) a similar pattern was obtained by incubating a KCl-supernatant of rat-liver homogenate with purified PAP^{35}S, (2) after hydrolysis of the incubated liver preparation with N-HCl for 15 min. or with the digestive juice of *Helix pomatia* the only spot revealed by chromatography was $^{35}SO_4^{2-}$.

The six unknown radioactive spots having the highest R_F values in the acetone:water medium were eluted off and examined spectroscopically. The spectra were somewhat masked by non-specific absorption but all showed a peak in the 275–280 region and strong absorption at lower wavelengths. This suggests that the spots are those of arylsulphates particularly since the digestive juice of *H. pomatia* is known to contain a potent arylsulphatase (Henry, Thevenet & Jarrige, 1952). Furthermore, the addition of an ether extract of the KCl-supernatant of rat liver to the incubation medium greatly increased the intensity of many of the spots particularly those with high R_F values in acetone:water. One of the spots has been identified as *p*-cresylsulphate.

The ester sulphate patterns of urine and bile from male and female rats injected with $Na_2{}^{35}SO_4$ were different from those of the liver preparation but some common spots were observed.

REFERENCES

Brunngraber, E. G. (1958). *J. biol. Chem.* **233**, 472.
Henry, R., Thevenet, M. & Jarrige, P. (1952). *Bull. Soc. Chim. biol., Paris*, **34**, 897.
Robbins, P. W. & Lipmann, F. (1957). *J. biol. Chem.* **229**, 837.

The Detection of Carbohydrates with Silver in the Presence of Borate. By H. G. BRITTON (introduced by A. ST G. HUGGETT). (*Department of Physiology, St Mary's Hospital Medical School, London, W.* 2)

The chromatogram paper is dipped in a solution of 40% hydrogen fluoride (1–4 vol.) and acetone (40 vol.) ensuring that the paper is well saturated. After allowing 5–10 min. at room temperature the paper is developed with silver following the method of Trevelyan, Procter & Harrison (1950). It is dipped in a solution made of saturated silver nitrate in water (0·1 vol.) and acetone (20 vol.) to which water has been added drop by drop until the precipitate has redissolved. When dry it is sprayed with a solution of sodium hydroxide, 50% in water (5 vol.) and ethyl alcohol (45 vol.) After the spots have fully developed the paper is treated with 2N-ammonia until the best contrast is obtained and then washed for at least an hour.

The amount of hydrogen fluoride required depends on the quantity of borate present: for example 2 vol. were needed for an electrophoresis strip run in 0·05M-borax. With these modifications the sensitivity of the silver method is comparable to that in the absence of borate.

I would like to thank Dr J. E. Scott of the Surgical Unit, St Mary's Hospital, for his help with this work.

REFERENCE

Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950). *Nature, Lond.*, **166**, 444.

Structure of Glycogen and Amylopectin Muscle-Phosphorylase Limit Dextrins. By GWEN J. WALKER and W. J. WHELAN. (*The Lister Institute, Chelsea Bridge Road, London, S.W.* 1)

Cori & Larner (1951) examined the structure of the rabbit-muscle-phosphorylase limit dextrins (P-dextrins) of glycogen and amylopectin. They suggested that the side chains (*A* chains, Peat, Whelan & Thomas, 1952) were single α-glucose units joined through 1:6-links to the main chains (*B* chains), the outer portions of which were 5–6 units long, or 6–7 units (Larner, Illingworth, Cori & Cori, 1952). The following evidence is inconsistent with this picture.

(1) R-Enzyme, the amylopectin-debranching enzyme (Hobson, Whelan & Peat, 1951), does not hydrolyse 1:6-linked α-glucose residues of the type postulated in P-dextrin (Roberts, 1953; Whelan, 1953). Its action on waxy-maize amylopectin P-dextrin, as measured by subsequent β-amylolysis, was far greater than could be accounted for from the Cori type of molecule.

(2) The smallest maltodextrin formed by the R-enzyme was maltotetraose. It was not formed from the parent amylopectin. The smallest maltodextrin liberated from the Cori P-dextrin would be maltohexaose.

(3) Salivary α-amylase acting on glycogen or amylopectin in dilute concentration liberates 6^3-α-maltosylmaltotriose as the smallest branched oligosaccharide (Whelan & Roberts, 1952; Whelan & Bines, 1955). In high concentration the smallest molecule liberated is 6^3-α-glucosylmaltotriose (Nordin & French, 1958). This latter molecule should be formed from the Cori P-dextrin by the dilute amylase since the α-glucosyl residue of the tetrasaccharide is preformed in the dextrin. The P-dextrins of rabbit-liver and oyster glycogen and of waxy-maize amylopectin fail to give the tetrasaccharide with dilute enzyme but do so with concentrated amylase.

The evidence, taken with the extent of action of β-amylase on the outer chains of P-dextrin (Hestrin, 1949), is consistent with the *A* chains and the outer *B* chains of the P-dextrin each being 4 units long. This revision of the structure necessitates a re-investigation of the action of amylo-1:6-glucosidase. The enzyme liberates glucose from the dextrin, this glucose being supposed to represent the *A* chains (Cori & Larner, 1951). It has been suggested (Whelan, 1958) that glucose would be formed if the glucosidase were contaminated with an α-1:4-glucosyl transferase, known to be present in animal tissue (Giri, Nagabhushanam, Nigam & Belavadi, 1955; Petrova, 1958; Stetten, 1959). Amylo-1:6-glucosidase purified as by Cori (1955) has now been found to contain this transferase.

We thank the Agricultural Research Council and the Rockefeller Foundation for grants.

REFERENCES

Cori, G. T. (1955). In *Methods in Enzymology*, vol. 1, p. 211. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Cori, G. T. & Larner, J. (1951). *J. biol. Chem.* **188**, 17.
Giri, K. V., Nagabhushanam, A., Nigam, V. N. & Belavadi, B. (1955). *Science*, **121**, 898.
Hestrin, S. (1949). *J. biol. Chem.* **179**, 943.
Hobson, P. N., Whelan, W. J. & Peat, S. (1951). *J. chem. Soc.* p. 1451.
Larner, J., Illingworth, B., Cori, G. T. & Cori, C. F. (1952). *J. biol. Chem.* **199**, 641.
Nordin, P. & French, D. (1958). *J. Amer. chem. Soc.* **80**, 1445.
Peat, S., Whelan, W. J. & Thomas, G. F. (1952). *J. chem. Soc.* p. 4546.
Petrova, A. N. (1958). *Abstr. 4th int. Congr. Biochem., Vienna*, 4–123.
Roberts, P. J. P. (1953). Ph.D. Thesis, University of Wales.
Stetten, M. (1959). *J. Amer. chem. Soc.* **81**, 1437.
Whelan, W. J. (1953). *Symp. biochem. Soc.* **11**, 17.
Whelan, W. J. (1958). In *Lectures on the Scientific Basis of Medicine*. 1956–1957, vol. 6, p. 246. London: Athlone Press.
Whelan, W. J. & Bines, B. J. (1955). *Biochem. J.* **61**, i.
Whelan, W. J. & Roberts, P. J. P. (1952). *Nature, Lond.*, **170**, 748.

The Metabolic Pathway of Phthalic Acid by Soil *Pseudomonas*. By D. W. RIBBONS and W. C. EVANS. (*Department of Agricultural Chemistry, University College of North Wales, Bangor*)

Evans (1955) detected by chromatography 4:5-dihydroxyphthalic acid as an early intermediate in the oxidative metabolism of phthalic acid by soil *Pseudomonas*. This metabolite and protocatechuic acid obeyed the criteria of sequential induction whilst no other mono- or di-hydroxyphthalic or benzoic acid did.

We have now isolated protocatechuic acid from phthalate cultures. A cell-free enzyme system prepared from phthalate-grown cells failed to produce 4:5-dihydroxyphthalic acid from phthalate, but readily decarboxylated this intermediate anaerobically to protocatechuic acid. This aromatic decarboxylase action may be followed mano-

metrically, in N_2/CO_2 atmosphere, whence one mole of CO_2 is released per mole of 4:5-dihydroxyphthalate, with the production of one mole of protocatechuate—which in the absence of oxygen is not further metabolized by the enzyme system. Ultraviolet spectrophotometry confirms this change.

This aromatic decarboxylase enzyme differs from those previously met with in this field, which are all oxidative decarboxylations. So far, no cofactor requirements have been demonstrated.

In the presence of oxygen, the dialysed cell-free enzyme system above produces β-carboxymuconic acid, from protocatechuic acid, whilst the undialysed preparation gives β-oxoadipic acid; this is in conformity with previous work (Parr, Evans & Evans, 1949; Stanier & Ingraham, 1954; MacDonald, Stanier & Ingraham, 1954).

REFERENCES

Evans, W. C. (1955). *Biochem. J.* **61**, x.

MacDonald, D. L., Stanier, R. Y. & Ingraham, J. L. (1954). *J. biol. Chem.* **210**, 809.

Parr, W. H., Evans, R. A. & Evans, W. C. (1949). *Biochem. J.* **45**, xxix.

Stanier, R. Y. & Ingraham, J. L. (1954). *J. biol. Chem.* **210**, 799.

The Pathway of Metabolism of Protocatechuic Acid by *Pseudomonas* species. By D. W. Ribbons and W. C. Evans. (*Department of Agricultural Chemistry, University College of North Wales, Bangor*)

Gross, Gafford & Tatum (1956), using [2:6-$^{14}C_2$]-protocatechuic acid as substrate, found that a cell-free enzyme system from *Neurospora crassa* metabolized it to β-oxoadipic acid, according to the following sequence of reactions:

Protocatechuic acid → *cis–cis*-β-carboxymuconic acid → (−)-β-carboxymuconolactone → β-oxoadipic acid.

Stepwise degradation of the β-oxoadipic acid so formed showed the keto group C to be derived solely from C-6 of protocatechuic acid.

A *Pseudomonas* cell-free enzyme system acting on the same substrate also gave β-oxoadipic acid, but on degradation the keto group C was now randomly derived from C-1 and C-6 of protocatechuic acid. Moreover, this system was without activity on (+)- or (−)-β-carboxymuconolactone, thus excluding it from being an intermediate. The known members of this pathway are:

Protocatechuic acid → *cis–cis*-β-carboxymuconic acid → β-oxoadipic acid.

Clearly, the *Pseudomonas* pathway diverges from the *Neurospora* route after the *cis–cis*-β-carboxymuconic acid stage, although the end product is the same.

The tracer results imply that in the *Pseudomonas* pathway an intermediate possessing a symmetrical structure is formed before β-oxoadipic acid. Butanolido-βγ-γ′β′-butanolide (2:6-dioxa-3:7-dioxo-*bicyclo*[3:3:0]octane, or dilactone, (abb.)), or its open-chain counterpart ββ′-dihydroxyadipic acid is a possibility; dehydrase action on this would produce β-oxoadipic acid with the keto group C derived equally from β- and β′-carbon atoms.

To test this hypothesis, the dilactone was synthesized by the method of Elvidge, Linstead, Sims & Orkin (1950), and used as substrate in parallel with other likely intermediates, with both enzyme systems. β-Oxoadipic acid production was detected by the Rothera reaction, estimated by catalytic decarboxylation, and confirmed by chromatography of its 2:4-dinitrophenylhydrazone. The *Pseudomonas* cell-free system produced β-oxoadipic acid when the 'dilactone' was used as substrate, but the *Neurospora* system failed to do so.

The evidence supports the contention that the dilactone or its corresponding ββ′-dihydroxyadipic acid is the intermediate preceding β-oxoadipic acid in the metabolism of protocatechuic acid by these *Pseudomonas*.

It is relevant to record that Landa & Eliasek (1956) isolated this dilactone from culture fluid of *Oospora* spp. metabolizing catechol; they thought it to be an artifact produced from γ-carboxymethylbutenolide during isolation, but it may well be a real intermediate.

REFERENCES

Elvidge, J. A., Linstead, R. P., Sims, P. & Orkin, B. A. (1950). *J. chem. Soc.* p. 2235.

Gross, S. R., Gafford, R. D. & Tatum, E. L. (1956). *J. biol. Chem.* **219**, 781.

Landa, S. & Eliasek, J. (1956). *Chem. Listy*, **50**, 1834.

Metabolism of 2:4-Dichlorophenoxyacetic acid by Soil *Pseudomonas*: Isolation of α-Chloromuconic Acid as an Intermediate. By H. N. FERNLEY and W. C. EVANS. (*Department of Agricultural Chemistry, University College of North Wales, Bangor*)

A strain of *Pseudomonas* species originally isolated from conifer litter (Evans & Smith, 1954) was grown in a mineral-salts medium containing up to 0·1% 2:4-dichlorophenoxyacetic acid (2:4-D) as sole carbon source. Towards the end of the logarithmic phase of growth the ultraviolet absorption peak of 2:4-D (283 mμ) is replaced by another at 272 mμ, which persists for some time. From 80 l. of culture medium at this stage, 200 mg. of an acidic product, m.p. 160°, $\lambda_{max.}$ 277 mμ (ethanol) was obtained; maximum yields under the most favourable conditions were estimated at 4 mg./l. of culture fluid (microanalysis gave C, 40·71; H, 3·06; Cl, 20·3. $C_6H_5O_4Cl$ requires C, 40·8; H, 2·83; Cl, 20·1%). Microtitration indicated a dibasic acid, equivalent weight, 88. On catalytic hydrogenation, 3 μmoles of hydrogen were absorbed per μmole of the acid (based on a molecular weight of 176·5). The evidence agrees with a monochloromuconic acid. Oxidation of *o*-chlorophenol with peracetic acid (Boeseken & Metz, 1935) gave α-chloromuconic acid (m.p. 160°) identical with the culture isolate (mixed m.p. undepressed).

In contrast with the β-chloromuconic acid isolated from 4-chlorophenoxyacetic acid cultures (Evans & Moss, 1957), both the synthetic and culture-isolated α-chloromuconic acids are readily metabolized by 2:4-D-grown cells, with absorption of oxygen and liberation of chloride ions. Of the four possible isomers therefore, this compound probably has the *cis-cis* configuration. Cultures of 2:4-D accumulate under certain conditions a Folin positive substance, which was steam volatile and was shown to be 2:4-dichlorophenol (tosyl deriv.).

It appears likely that the other chloro-substituent of 2:4-D is eliminated from the aromatic ring.

The Agricultural Research Council are thanked for their support of this work.

REFERENCES

Boeseken, J. & Metz, C. F. (1935). *Rec. trav. chim. Pays-Bas*, **54**, 345.

Evans, W. C. & Moss, P. (1957). *Biochem. J.* **65**, 8P.

Evans, W. C. & Smith, B. S. W. (1954). *Biochem. J.* **57**, xxx.

(The Editors of the Biochemical Journal accept no responsibility for the Reports of the Proceedings of the Society)

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **386th Meeting** *of the Society was held in conjunction with the second Scandinavian Summer Meeting of Biochemists, Pharmacologists, and Physiologists in Turku (Åbo), Finland from Thursday, 27 August to Saturday, 29 August. The following papers were read by members of The Biochemical Society:*

* Indicates that the member received a grant from the Travel Fund of the Society.

COMMUNICATIONS

The Use of a Perfusion Apparatus to Study Continuously the Transport of ^{131}I-Labelled Iodide by the Small Intestine.

By J. D. Acland. (*Department of Pharmacology and Therapeutics, The University, Sheffield* 10, *England*)

In rats, the central region of the small intestine has been shown to transport I against a concentration gradient into its lumen *in vivo* (Pastan, 1957) and from the serosal to the mucosal side of everted sacs *in vitro* (Acland & Illman, 1959). In each of the present series of experiments, everted segments of jejunum taken from three rats were perfused together in a modified form of the apparatus described by Wiseman (1953). The mucosal and serosal perfusion fluids (Krebs–Ringer bicarbonate or phosphate buffer containing ^{131}I-labelled KI) were circulated through flow-through Geiger–Müller tubes (Type F. 10, 20th Century Electronics Ltd.) connected to a scaler specially constructed from 'Add-on' units (Ericsson Telephones Ltd.). Simultaneous radioactivity determinations on the serosal and mucosal fluids were used to follow changes in their I′ concentrations.

When the initial I′ concentration was 1 μM, mucosal/serosal I′ concentration ratios ($[I']_M/[I']_S$) of 3–8 were built up and maintained in 4 experiments lasting up to 9 hr. Both gassing with N_2 (3 experiments) and adding $KClO_4$ (4 experiments) to the serosal fluid (final concentration 0·1 mM) caused the ratio $[I']_M/[I']_S$ to fall within 20–40 min. When KCN (3 experiments) was added to the serosal fluid (final concentration 4 mM), the ratio $[I']_M/[I']_S$ did not decrease for 1–2 hr., on one occasion even continuing to rise during this period, although at a slower rate. In 2 experiments, methylthiouracil added to the serosal fluid (final concentration 1·5 mM) appeared to be without effect on iodide transport.

It seems unlikely in the light of these results, either that there is a barrier to the passage of iodide across the intestinal mucosa or that iodide is strongly bound to a protein secreted by the mucosal cells. Oxidation of iodide is probably not concerned in its transport by the intestine against a concentration gradient. Cyanide may possibly compete with iodide for a protein carrier while also inhibiting active iodide transport through its effect on respiratory enzymes.

It is suggested that the apparatus described could be used generally in perfusion studies on labelled compounds.

REFERENCES

Acland, J. D. & Illman, O. (1959). *J. Physiol.* (in the Press).
Pastan, I. (1957). *Endocrinology*, **61**, 93.
Wiseman, G. (1953). *J. Physiol.* **120**, 63.

Changes in the Metabolism of DL-[β-^{14}C]Serine and [^{14}C]Formate in the Vitamin B_{12}-Deficient Rat.

By H. R. V. Arnstein.* (*National Institute for Medical Research, Mill Hill, London, N.W.* 7, *England*)

The metabolic function of vitamin B_{12} in the interconversion of C_1 metabolites has been studied *in vivo* and *in vitro*, using young albino rats which had been depleted of vitamin B_{12} by breeding from mothers fed on the same vitamin B_{12}-deficient diet. A diet containing 12% ethanol-extracted casein (vitamin B_{12} content < 1 μmg./g.) was used in most experiments, control rats receiving 1–3 μg. of vitamin B_{12}/day. On this diet, the vitamin B_{12} content of liver and brain was decreased to 10–20% of the controls. Some litters showed a high mortality soon after birth, but survivors grew well.

Vitamin B_{12} deficiency had no effect on the incorporation of ^{14}C-labelled serine and histidine into the mixed visceral proteins. The conversion of formate (either fed or injected) into choline was, however, markedly decreased. A similar effect was observed with a soya-lactose diet (Cuthbertson & Thornton, 1952). The conversion of formate into both serine and methionine was also reduced, as shown by a decrease in the specific radioactivity of serine from 76·0 to 42·3 and of methionine from 84·0 to 52·5 μc/mole.

The incorporation of fed or injected formate into RNA purines was greater in deficient animals. For example, 24 hr. after injection of sodium

[^{14}C]-formate (4·35 μc/rat) the purines of liver RNA had the following activities (μmc/g.): adenine picrate, 144 ($+B_{12}$), 264 ($-B_{12}$); guanine sulphate, 138 ($+B_{12}$), 168 ($-B_{12}$). Vitamin B_{12} added *in vitro* to minces of liver or spleen from deficient animals fed on an α-protein diet (Henry & Porter, 1958) had, however, no effect on the conversion of labelled formate into the nucleic acids. It is possible that the 2 or 3 hr. incubation periods used in these experiments were too short for vitamin B_{12} to become effective.

The utilization of DL-(β-^{14}C]serine *in vivo* for the biosynthesis of choline methyl groups was increased by vitamin B_{12} to a similar extent as that of formate. In contrast to formate, however, vitamin B_{12} deficiency slightly decreased the conversion of DL-[β-^{14}C]serine into RNA purines.

These experiments indicate that, *in vivo*, vitamin B_{12} increases the reduction of formate to the hydroxymethyl group of serine, as well as to the methyl groups of choline and methionine, and the oxidation of the hydroxymethyl group of serine to formate. In these reactions, the active metabolites are probably derivatives of folic acid and it is suggested that vitamin B_{12} functions in the oxidation–reduction of these intermediates but not in their transfer at the same oxidation level.

REFERENCES

Cuthbertson, W. F. J. & Thornton, D. M. (1952). *Brit. J. Nutr.* **6**, 170.

Henry, K. M. & Porter, J. W. G. (1958). *Proc. Nutr. Soc.* **17**, vii.

Studies on the Synthesis of Serum Albumin by Liver Microsomes. By P. N. CAMPBELL and BARBARA A. KERNOT. (*Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W. 1, England*)

Previous studies on the possible synthesis of serum albumin by the isolated microsome fraction from regenerating rat liver have been continued (Campbell, Greengard & Kernot, 1958). In the initial experiments the microsomes were incubated with cell sap, energy source (phosphoenolpyruvate) and radioactive amino acids, and an energy-dependent incorporation of amino acids into the serum albumin released from the microsome pellet was demonstrated. Similar results have now been obtained with microsomes from normal rat liver.

In the above experiments the identity of the serum albumin rested solely on immunological evidence. Larger amounts of microsomes from regenerating liver have therefore been incubated with radioactive amino acids and the albumin released from the microsomes by ultrasonics characterized first by purification by the trichloroacetic acid–ethanol procedure of Debro, Tarver & Korner (1957) and then by zone electrophoresis on cellulose acetate paper. The serum albumin was finally precipitated by the addition of a specific antiserum. Although the application of these procedures led to some reduction in the initial radioactivity of the isolated serum albumin, the microsomal albumin remained significantly radioactive throughout purification.

Similar procedures were applied to the albumin present in the supernatant obtained after centrifuging the incubation mixture. In this case the albumin obtained after electrophoresis was not radioactive.

These results are consistent with the synthesis of serum albumin by the isolated microsome fraction from rat liver but suggest that the newly synthesized albumin is not released in a soluble form. A comparison of the radioactivity of the microsomal albumin with that of the insoluble proteins obtained from regenerating liver microsomes labelled *in vivo* and *in vitro* suggests a relatively greater loss in the ability of the isolated microsomes to synthesize albumin as opposed to structural protein (Campbell, Greengard & Kernot, 1959).

In order to determine the role of the cell sap in the synthesis of specific proteins by liver microsomes, washed microsomes from regenerating rat liver were incubated in the presence of duck-liver cell sap (Campbell & Kernot, 1959). The ratio of the specific radioactivity of the microsomal rat albumin to insoluble microsomal protein was not significantly affected by the substitution of the heterologous for the homologous cell sap. No evidence of the synthesis of duck serum albumin by rat-liver microsomes was obtained. These results suggest that the cell sap is without influence on the immunological properties of the serum albumin synthesized by the microsomes.

REFERENCES

Campbell, P. N. & Kernot, B. A. (1959). *Biochem. J.* **72**, 6P.

Campbell, P. N., Greengard, O. & Kernot, B. A. (1958). *Biochem. J.* **68**, 18P.

Campbell, P. N., Greengard, O. & Kernot, B. A. (1959). *Biochem. J.* In the press.

Debro, J. R., Tarver, H. & Korner, A. (1957). *J. Lab. clin. Med.* **50**, 728.

Mechanism of the Glyoxalase I Reaction and Competitive Inhibition by Ophthalmic Acid. By E. E. CLIFFE* and S. G. WALEY. (*Nuffield Laboratory of Ophthalmology, University of Oxford, England*)

The overall reaction catalysed by glyoxalase I is the conversion of methylglyoxal and glutathione into *S*-lactoylglutathione:

$$CH_3 \cdot CO \cdot CHO + GSH \rightleftarrows CH_3 \cdot CH(OH) \cdot CO \cdot SG \quad (1)$$

Methylglyoxal and glutathione combine non-enzymically to form a labile hemithioacetal [$CH_3 \cdot CO \cdot CH(OH) \cdot SG$] and all three compounds are present at equilibrium. The question arises whether the enzyme combines with the free methylglyoxal and the free glutathione or with the hemithioacetal.

When methylglyoxal and glutathione were allowed to react together until the hemithioacetal had reached its equilibrium concentration, and the enzyme then added, the initial velocities of a series of enzymic reactions were symmetrical functions of the concentrations of methylglyoxal and glutathione. This is consistent with the hemithioacetal being the substrate (see Segal, Kachmar & Boyer, 1952), which would mean that the first stage in reaction (1) is the formation of the hemithioacetal. So the rates of formation of the hemithioacetal have been measured under various conditions and this information has been used in the investigation of the overall reaction.

The hypothesis that the hemithioacetal is the substrate for the reaction has been tested in experiments in which reaction (1) was started by adding methylglyoxal to the enzyme and glutathione. Under these conditions no hemithioacetal was present initially. The course of the enzymic reaction was predicted from a knowledge of the course of the hemithioacetal formation and of the maximum velocity and the Michaelis constant obtained from the series of reactions in which the hemithioacetal was allowed to form before the enzymic reaction was started. The observed and predicted courses of the enzymic reaction were in good agreement.

The mechanism of the reaction can be written:

$$\text{Methylglyoxal} + \text{glutathione} \rightleftarrows \text{hemithioacetal}$$

$$\text{Hemithioacetal} + \text{glyoxalase I} \rightleftarrows \text{enzyme–substrate complex} \rightarrow S\text{-lactoylglutathione} + \text{glyoxalase I}$$

It is unusual in that it involves the compulsory formation of a binary complex before enzymic reaction can occur and the glyoxalase reaction seems to be the first example of this type of mechanism.

These findings are at variance with the work of Kermack & Matheson (1957), who suggested that glyoxalase I reacts with free methylglyoxal and free glutathione.

Ophthalmic acid, a naturally occurring analogue of glutathione (Waley, 1958), was found to inhibit competitively in the reaction and an inhibitor constant (K_i) has been determined.

REFERENCES

Kermack, W. O. & Matheson, N. A. (1957). *Biochem. J.* **65**, 48.

Segal, H. L., Kachmar, J. F. & Boyer, P. D. (1952). *Enzymologia*, **15**, 187.

Waley, S. G. (1958). *Biochem. J.* **68**, 189.

The Incorporation of Amino Acids into a Ribonucleoprotein Fraction of Microsomes: a Possible Pathway of Protein Synthesis. By P. COHN.* (*Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, London, S.W. 3, England*)

Several laboratories have now confirmed the existence of a system capable of incorporating amino acids into proteins (Hultin & Beskow, 1956; Keller & Zamecnik, 1956; Simkin & Work, 1957) which consists of (*a*) the microsome fraction of cell homogenates, (*b*) the so-called 'pH 5 sediment' together with polynucleotides, (*c*) an energy-generating system and (*d*) Mg^{2+} and K^+ ions. Recent results from this laboratory (Cohn, 1959) indicate that even when an appreciable part of the protein and lipids of the microsomes has been removed by treatment with certain detergents, the residual ribonucleoprotein material is still capable of incorporating amino acids, at least to the same extent as the microsomes. This incorporation, however, occurs equally well in the absence of added 'pH 5 sediment' or of the cell sap.

In these experiments a non-ionic detergent (Lubrol W) is used to act on the microsomes of rat liver. The insoluble sediment is then partially solubilized by the addition of sodium perfluorooctanoate. It was the protein of this final pellet which on incubation with [^{14}C]amino acids in the presence of a system generating adenosine triphosphate usually showed a higher level of radioactivity than that of the Lubrol pellet or of the intact microsomes. The addition of cell sap stimulated the incorporation of amino acids into the protein of microsomes and, on most occasions, of the Lubrol pellet.

Further experiments have been made which confirm and extend this finding. Some of the requirements for co-factors in the incorporation into the residual ribonucleoprotein were examined; attempts were also made to investigate possible intermediate steps in this system. A detectable but small proportion of the radioactive amino acid was observed to be extracted with the ribonucleic acid by phenol. The remainder is firmly associated with the protein, but it has not yet been demonstrated that it is linked through peptide bonds.

The presence of activating enzymes in the perfluoro-octanoate pellet could not be detected when the exchange of [^{32}P]pyrophosphate with ATP was examined. It appears, therefore, that the preparation of this ribonucleoprotein fraction has uncovered a pathway, different from that for whole microsomes, leading to protein synthesis.

REFERENCES

Cohn, P. (1959). *Biochim. biophys. Acta*, **33**, 284.
Hultin, T. & Beskow, G. (1956). *Exp. Cell Res.* **11**, 664.
Keller, E. B. & Zamecnik, P. C. (1956). *J. biol. Chem.* **221**, 45.
Simkin, J. L. & Work, T. S. (1957). *Biochem. J.* **67**, 617.

Amines in Blood and Urine. By A. M. Asatoor and C. E. Dalgliesh. (*Department of Chemical Pathology, Postgraduate Medical School, London, W. 12, England*)

In diseases of the liver detoxication mechanisms may be disturbed with possible accumulation in the blood of potentially toxic substances such as amines. We have therefore compared the level of amines in normal subjects and patients with liver diseases. A method, based on that described by McIntire, Clements & Sproull (1953), has been developed for the accurate estimation of very small quantities of amines in blood and urine.

After precipitation of proteins in whole blood, diluted with water, the supernatant is treated with 1-fluoro-2:4-dinitrobenzene. Excess of reagent is converted into sodium 2:4-dinitrophenate. DNP-Amines are extracted with monochlorobenzene and the absorption of the extract is measured at 365 mμ, using 4 cm. cells.

Ammonia present in urine is removed by treatment with yellow mercuric oxide (Francois, 1907) and, after spinning, the supernatant is similarly treated with fluorodinitrobenzene. DNP-Amines are extracted with *cyclo*hexane and the absorption measured at 350 mμ.

Blood from 30 normal subjects gave a mean value of 0·41 ± S.D. 0·09 (range 0·25–0·67) expressed as μg. of N/ml. of blood, using β-phenylethylamine as standard. Blood from 26 patients with liver diseases gave a mean of 0·53 ± S.D. 0·14 (range 0·17–1·00).

Amines were estimated in urine from 20 normal subjects and 20 patients. Normals: mean 8·4 ± S.D. 2·15 (range 5·1–12·9) expressed as mg. of N in 24 hr. using piperidine as standard. Patients: mean 6·7 ± 1·7 (range 4·0–11·1).

Reversed-phase paper chromatography gave the most satisfactory results for identification of the individual DNP-amines. Four types of paper were used: (1) Silicone-treated paper, using as solvent the upper phase of a mixture of chloroform, methanol and water (10:10:6) (Kritchevsky & Tiselius, 1951; Lockhart, 1956). (2) Paraffin-treated paper, using as solvent a chloroform, methanol, water, liquid paraffin mixture (10:10:6:4) which had been allowed to stand for about 8 hr. (3) Acetylated paper (Zijp, 1955). (4) Benzoylated paper (Micheel & Albers, 1956). For general use paraffin-treated paper was found most satisfactory. Methylamine, dimethylamine, ethylamine, pyrrolidine and piperidine were found in normal and pathological urines.

In one patient *iso*amylamine and β-phenylethylamine were detected in blood.

We thank Dr Sheila Sherlock and colleagues for their helpful co-operation.

REFERENCES

Francois, M. (1907). *C.R. Acad. Sci., Paris*, **144**, 567.
Kritchevsky, T. H. & Tiselius, A. (1951). *Science*, **114**, 299.
Lockhart, I. M. (1956). *Nature, Lond.*, **177**, 393.
McIntire, F. C., Clements, L. M. & Sproull, M. (1953). *Anal. Chem.* **25**, 1757.
Micheel, F. & Albers, P. (1956). *Chem. Ber.* **89**, 140.
Zijp, J. W. H. (1955). *Chem. Weekbl.* **51**, 547.

Studies in the Incorporation of [*Me*-^{14}C]Choline and L-[^{14}C]Serine into the Phospholipids of Subcellular Particles of Rat Liver. By R. R. Dils* and G. Hübscher. (*Department of Medical Biochemistry and Pharmacology, The Medical School, Birmingham 15, England*)

Isotope tracer studies by Kennedy and co-workers (1957) have elucidated the steps in the biosynthesis *in vitro* of phosphatidylcholine from labelled phosphorylcholine. In earlier experiments, however, where labelled choline had been used (Kennedy, 1954), the radioactive lipid subsequently isolated was

tentatively identified as a fatty acid ester of choline and not phosphatidylcholine (Kennedy, 1956*a*, *b*).

Work on the incorporation of [*Me*-^{14}C]choline into the lipids of subcellular particles of rat liver showed that cytidine monophosphate stimulated the incorporation (Dils & Hübscher, 1959), as opposed to Kennedy's findings (1957) that cytidine triphosphate was required for the incorporation of labelled phosphorylcholine.

In view of this difference, the lipid obtained from labelled choline was analysed to determine whether it was phosphatidylcholine or a fatty acid ester of choline. The lipid was purified by silicic acid chromatography and then hydrolysed by mild alkali. This splits phospholipids to their diesters (e.g. phosphatidylcholine to glycerylphosphorylcholine) and choline esters of long-chain fatty acids to free choline. The hydrolysis products were then separated by column chromatography on Dowex 1 (Hawthorne & Hübscher, 1959).

Of the activity recovered from the resin column, 92·6% appeared in the eluent containing glycerylphosphorylcholine and any free choline possibly present. These were then separated by paper chromatography and their activities determined by liquid scintillation-counting. 77·4–84·5% of the activity recovered was associated with the glycerylphosphorylcholine spot, indicating that most of the labelled choline used in these experiments had been incorporated into the phosphatidylcholine of the subcellular fractions of rat liver.

The significance of the co-factors required for this incorporation (cytidine monophosphate, coenzyme A, glutathione and adenosine triphosphate) will be discussed.

Previous experiments (Dils & Hübscher, 1959) have shown that small amounts of Ca^{2+} ions strongly stimulate this incorporation, even in the absence of the co-factors listed above. Stimulation by calcium ions alone also occurs when labelled serine is incorporated into phosphatidylserine under similar circumstances (Hübscher, Dils & Pover, 1959).

This calcium-activated system has the following properties: optimum calcium concentrations of 1·6 mM for choline and 15 mM for serine; optimum pH at 9·0 (choline) and 8·5 (serine). As with choline, calcium could not be replaced by other bivalent metals when serine was used. Magnesium, in fact, inhibited the incorporation due to calcium with both choline and serine. Unlabelled L-serine reduced the incorporation of labelled choline, though the system involved in the serine incorporation was significantly more stable to freezing and thawing than that involved in the choline incorporation.

The calcium-stimulated incorporations of choline and serine could be explained by exchange reactions catalysed by a phospholipase D, though this has only been described in plants.

REFERENCES

Dils, R. R. & Hübscher, G. (1959). *Biochim. biophys. Acta*, **32**, 293.

Hawthorne, J. N. & Hübscher, G. (1959). *Biochem. J.* **71**, 195.

Hübscher, G., Dils, R. R. & Pover, W. F. R. (1959). *Biochim. biophys. Acta* (in the Press).

Kennedy, E. P. (1954). *J. biol. Chem.* **209**, 525.

Kennedy, E. P. (1956*a*). *Canad. J. Biochem. Physiol.* **34**, 334.

Kennedy, E. P. (1956*b*). *J. biol. Chem.* **222**, 193.

Kennedy, E. P. (1957). *Ann. Rev. Biochem.* **26**, 119.

Observations on the Metabolic Fate of the Sulphate Esters of Phenols. By K. S. Dodgson* and N. Tudball. (*Department of Biochemistry, University of Wales, Newport Road, Cardiff, Wales*)

Certain tissues, particularly liver, can accomplish the esterification of phenols with sulphuric acid to give arylsulphates. The enzyme systems responsible for this synthesis are located in the cell cytoplasmic fluid. Also present in tissues are three distinct arylsulphatases of differing specificity. In the liver cell two of these enzymes are localized mainly in the mitochondria whilst the other is microsomal in origin. The three enzymes are widely distributed in tissues but their physiological role is obscure. It is clear, however, that they are not identical with the enzymes concerned in the direct synthetic process (see Baum & Dodgson, 1958). It is possible of course that the existence of a phenol-sulphating system in the cell fluid and the presence of different arylsulphatases in intracellular particles may be a relationship of some significance and this point will be discussed.

From the few studies which have been made on the metabolic fate of arylsulphates it would appear, at first sight, that the esters of phenols which can be regarded as 'non-physiological' (e.g. phenyl and naphthyl sulphates) are not appreciably hydrolysed *in vivo* (Sperber, 1948; Garton & Williams, 1949; Hawkins & Young, 1954). In contrast, esters of physiologically active phenols (e.g. oestrone sulphate, 3:5:3′-tri-iodothyronine sulphate) are apparently hydrolysed *in vivo* (Hanahan & Everett, 1950; Michel, Roche, Closon & Michel, 1958). However, there are reasons, which can be deduced from enzyme specificity studies, for suspecting the limitations of such a differentiation.

Recently, we have begun investigations on the metabolic fate of arylsulphates in order to explore the possibility that such compounds, in association with the arylsulphatases, may play some important physiological role. For reasons which will be explained, *p*-nitrophenyl [^{35}S]sulphate ($NP^{35}S$) and tyrosine *O*-[^{35}S]sulphate have been selected for initial studies although, at the present time, complete details are available for $NP^{35}S$ only. The ester sulphate group of intraperitoneally injected $NP^{35}S$ undergoes a number of metabolic transformations in both male and female rats. Irrespective of the dose level, the bulk of the ^{35}S appears in the urine within 24 hr. Approximately 25% of the radioactivity of the initial dose is present in the urine as inorganic sulphate, 1–3% appears in the neutral sulphur fraction and the remainder as ester sulphate. Autoradiography of chromatograms of urine show the presence of ten ^{35}S-containing compounds in addition to inorganic sulphate and $NP^{35}S$. All these compounds appear to be ester sulphates (either aryl- or steroid-) since they are readily desulphated by mild hydrolysis with HCl or by treatment with the digestive juice of *Helix pomatia*. In many respects, the chromatographic pattern coincides with that obtained by similar treatment of the urine of rats receiving intraperitoneal injections of $Na_2{}^{35}SO_4$.

About 4% of the total radioactivity of the initial dose of $NP^{35}S$ is still present in the tissues after 48 hr. This is distributed in plasma, spleen, pancreas, kidney, liver, heart, skin and trachea. Chondroitin sulphate isolated from skin and from trachea contains ^{35}S, as also does the taurine fraction isolated from the liver. The two main metabolic routes by which these transformations can occur will be discussed.

This work was supported, in part, by a research grant (A. 1982) from the Arthritis and Metabolic Diseases Division of the U.S. Public Health Service.

REFERENCES

Baum, H. & Dodgson, K.S. (1958). *Nature, Lond.*, **181**, 115.
Garton, G. A. & Williams, R. T. (1949). *Biochem. J.* **45**, 158.
Hanahan, D. J. & Everett, N. B. (1950). *J. biol. Chem.* **185**, 919.
Hawkins, J. B. & Young, L. (1954). *Biochem. J.* **56**, 166.
Michel, R., Roche, J., Closon, J. & Michel, O. (1958). *Abstr. of Communications, IVth International Congress of Biochemistry*, sect. 12, no. 93.
Sperber, I. (1948). *K. LandtbrHogsk. Ann.* (*Ann. Roy. Agric. Coll., Sweden*), **15**, 317.

The Breakdown of Hyaluronic Acid in Rooster Comb. By J. Doyle and J. A. Szirmai. (*Research Laboratories, Department of Rheumatology, University Hospital, Leiden, The Netherlands*)

Much is known of hyaluronic acid biosynthesis, but little about its breakdown in biological systems. It is present in cock's comb in appreciable quantity (Boas, 1949). Szirmai (1956) demonstrated that the increase of metachromasia in comb connective tissue paralleled the increase in hexosamine. Schiller & Dorfman (1956) found hyaluronic acid and chondroitin sulphate in comb tissue in a ratio of 15:1.

Dissection of combs into fatty, mucoid, and epithelial layers was followed by isolation and identification of the mucopolysaccharides. The mucoid layer was relatively poor in sulphated material, while the fatty layer was relatively rich in this. This accords with the histological picture which shows association between vascularity and sulphated material, as judged by metachromatic staining at pH 2. Balazs & Szirmai (1958) found galactosamine in low concentration in isolated mucoid layer, so that studies of hyaluronic acid metabolism concern this layer.

To study the breakdown of the ground substance, fifteen White Leghorn capons were treated with testosterone propionate (1 mg./day). Then the hormone was withdrawn and the combs analysed at various stages during regression. The homogenized combs were extracted twice with 20 ml. of water/g. wet weight, and the extract separated by high-speed centrifuging. The extracts were analysed for hexosamine, uronic acid and nitrogen.

As the comb size fell, the viscosity of the extract dropped together with hexosamine and uronic acid concentrations (based on wet weight values), while non-hexosamine nitrogen concentrations rose. There is evidence that water concentrations are altering, but not in phase with hyaluronic acid.

In free electrophoresis (0·05M, veronal buffer) comb extract showed three peaks, one very fast and one of intermediate mobility. This is similar to the picture obtained in umbilical cord extracts. Recent evidence (Bettelheim, 1958) suggests that hyaluronic acid is strongly linked to protein. The steady rise in extractable protein concentration of the comb extract could reflect changes in the complex before the onset of the catabolic process.

REFERENCES

Balazs, E. A. & Szirmai, J. A. (1958). *J. Histochem. Cytochem.* **6**, 416.
Bettelheim, F. A. (1958). *Nature, Lond.*, **182**, 1301.
Boas, N. F. (1949). *J. biol. Chem.* **181**, 573.
Schiller, S. & Dorfman, A. (1956). *Proc. Soc. exp. Biol., N.Y.*, **92**, 100.
Szirmai, J. A. (1956). *Proc. Soc. exp. Biol., N.Y.*, **93**, 92.

The Mechanism of Glucuronide Formation: A Review. By G. J. DUTTON. (*Department of Biochemistry, Queen's College, Dundee (University of St Andrews), Scotland*)

Until recently the biochemical mechanism responsible for glucuronide formation in animals remained obscure. Early hypotheses derived this conjugated glucuronic acid from a directly oxidized glucose molecule, either free or bound already by glycosidic link to the aglycone; available evidence made so simple a process unlikely, but did indicate the basic necessity of carbohydrate. Later work added requirements for oxygen, phosphorus and energy, and suggested that the glucose carbon chain passed on unbroken to glucuronic acid. Subsequent use of homogenates and cell fractions has revealed the constituent reactions and their sequence.

The immediate donor of glucuronic acid to the aglycone is uridine diphosphate glucuronic acid (UDPglucuronic acid), which arises from oxidation of UDPglucose; this in its turn has needed both uridine triphosphate (and hence ATP) and glucose 1-phosphate (and hence glycogen or similar source):

(i) Uridine triphosphate + glucose 1-phosphate → UDPglucose + pyrophosphate;

(ii) UDPglucose + DPN → UDPglucuronic acid + reduced DPN;

(iii) Aglycone + UDPglucuronic acid → aglycone glucuronide + UDP.

By this means 'ether', 'ester' and *N* glucuronides of foreign or endogenous compounds can be formed. It is possible that one microsomal enzyme, UDP-transglucuronylase, mediates glucuronyl transfer to all substrates examined, though this is not yet certain.

Reaction (iii) occurs in livers of mammals, including man, of birds, frogs and trout; trout UDP-transglucuronylase appears much less active and more thermolabile. In the cat, whose glucuronide-forming capacity seems very small, reaction (ii) occurs in liver and kidney, but (iii) is negligible. In the several other mammals examined both (ii) and (iii) are significant in liver and kidney only towards foetal term, increasing to adult importance after birth; this is true development, not removal of inhibition. Since bilirubin is excreted as a glucuronide, neonatal 'physiological' jaundice may be from persistence of such foetal characteristics, though other pathways for bilirubin (in, for example, the cat) are probable.

Reactions (ii) and (iii) occur in mammalian liver, kidney cortex, gastro-intestinal mucosa and connective tissue, UDP-transglucuronylase from all these sites not yet appearing appreciably different though being less evident in the last two. Interestingly, guinea-pig foetal stomach actively forms glucuronides, possibly including that of bilirubin.

The above mechanism of glucuronide synthesis is important but it need not be unique, and evidence of other pathways will be discussed. The bearing of UDPglucuronic acid and associated enzymes on polysaccharide synthesis will be mentioned.

Autonomic Reversible Swelling in Heart-Muscle Mitochondria (Sarcosomes). By R. B. BEECHEY† and F. A. HOLTON. (*M.R.C. Experimental Radiopathology Research Unit, Hammersmith Hospital, London, England*)

Many workers have reported that both *in vivo* and *in vitro* mitochondria respond to chemical changes in their environment by swelling or shrinking (see Ernster & Lindberg, 1958, for review). Some of these observations relate to irreversible degenerative swelling in which the respiratory mechanism of the mitochondrion is irretrievably damaged. Where the swelling has been reversed successfully, this has been achieved by changing the chemical environment of the mitochondrion, e.g. by addition of Mn^{2+} and/or ATP (Chappell & Perry, 1954; Beyer, Ernster, Löw & Beyer, 1955; Lehninger, Ray & Schneider, 1959) or ADP (Packer, 1959).

We wish to report some further properties of a reversible swelling phenomenon observed in isolated heart-muscle sarcosomes (Holton, 1957). The process is reversed without addition of reagents. We refer to it as Autonomic Reversible Swelling (ARS). In a respiring suspension of sarcosomes showing this phenomenon, as dissolved oxygen becomes exhausted aerobic swelling stops and anaerobic shrinking begins within 1–2 sec. of completion of reduction of the respiratory chain. Cessation of swelling under anaerobic conditions is reminiscent of the properties of liver mitochondria (Hunter, Davis & Carlat, 1956; Lehninger & Ray, 1957). Its automatic reversal, which under our conditions is rapid and complete, appears to constitute a new observation. The familiar connexion between oxidative phosphorylation and mitochondrial morphology appears in a striking form in the following property of ARS. In a medium in which respiratory control could be demonstrated, ARS was always present. If conditions were altered so that respiratory control disappeared (e.g. by the addition of Mg^{2+}, by ageing or by addition of DNP) ARS ceased. Moreover, in ageing

† Present address: Department of Biochemistry, University of Southampton.

experiments respiratory control and ARS were lost together.

Since the net synthesis of ATP may continue efficiently in the absence of respiratory control (e.g. in the heart sarcosome, in the presence of Mg^{2+}), it appears that autonomic reversible swelling is linked only indirectly with the ability of the sarcosome to produce external ATP, and may be more directly dependent on an earlier intermediate in the metabolic pathway leading to phosphorylation. We think it possible that autonomic reversible swelling represents part of the physiological control mechanism proposed by Ernster (1956*a*, *b*), whereby the respiration of the cell (and possibly also the transfer of material between mitochondrion and cytoplasm) is controlled by reversible changes in mitochondrial structure.

REFERENCES

Beyer, R. E., Ernster, L., Löw, H. & Beyer, T. (1955). *Exp. Cell Res.* **8**, 586.

Chappell, J. B. & Perry, S. V. (1954). *Nature, Lond.*, **173**, 1094.

Ernster, L. (1956*a*). *Exp. Cell Res.* **10**, 721.

Ernster, L. (1956*b*). *The Enzymic Organization of Mitochondria and its role in the Regulation of Metabolic Activities in Animal Tissues.* Uppsala: Almqvist and Wiksell.

Ernster, L. & Lindberg, O. (1958). *Ann. Rev. Physiol.* **20**, 13.

Holton, F. A. (1957). *Biochem. J.* **66**, 37P.

Hunter, F. E., Davis, J. & Carlat, L. (1956). *Biochim. biophys. Acta*, **20**, 237.

Lehninger, A. L. & Ray, B. L. (1957). *Biochim. biophys. Acta*, **26**, 643.

Lehninger, A. L., Ray, B. L. & Schneider, M. (1959). *J. biophys. biochem. Cytol.* **5**, 97.

Packer, L. (1959). *Ann. N.Y. Acad. Sci.* **72**, 518.

Action of Mushroom Tyrosinase on [16-^{14}C]Oestrone. By P. H. Jellinck.* (*Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W. 1, England*)

It has recently been demonstrated that incubation of mammalian liver slices or mushroom tyrosinase with [16-^{14}C]oestrone results in the formation of water-soluble, ether-insoluble metabolites in high percentage yield (Jellinck, 1959). The present communication deals with the nature of the products formed by the phenol oxidase system.

[16-^{14}C]Oestrone (20 μg.) was incubated, under different conditions, with either mushroom extracts or a mushroom tyrosinase preparation and the percentage of the original radioactivity remaining in the aqueous medium, after extraction with ether, was determined. Yields of 80–90% were obtained under optimum conditions.

The formation of these ether insoluble metabolites was found to be completely abolished under anaerobic conditions and in the presence of cyanide, azide or carbon monoxide and also if the phenolic group was blocked by methylation. There was marked inhibition by ascorbic acid, glutathione and *p*-nitrophenol, but iodoacetate and *p*-chloromercuribenzoate had no such effect.

The addition of serum albumin or globulin or egg albumin greatly increased the yield of ether-insoluble products when less than optimum amounts of mushroom tyrosinase were used, showing that protein binding was taking place. Denatured protein was less effective. The findings that the water-soluble ^{14}C was non-dialysable and accompanied protein on precipitation with trichloroacetic acid is further evidence for the interaction of oestrone metabolites with protein. No radioactive CO_2 was evolved during the incubations and the products formed showed no marked oestrogenic activity.

These results therefore indicate that, in the presence of tyrosinase, there is oxidation of the aromatic ring of oestrone to one or more metabolites which then interact with protein, in a manner not involving sulphydryl groups, to form highly stable products. The latter type of reaction has been shown to take place with a number of quinones (Mason, 1955) and is probably non-enzymic.

REFERENCES

Jellinck, P. H. (1959). *Biochem. J.* **71**, 665.

Mason, H. S. (1955). *Advanc. Enzymol.* **16**, 105.

Protein Interactions which Condition Metabolic Response to Electrical Pulses in Mammalian Cerebral Tissues. By H. McIlwain.* (*Department of Biochemistry, Institute of Psychiatry, University of London, England*)

After keeping slices of cerebral cortex in buffered glucose media at 0° for 5–12 hr., the increase in respiration and glycolysis normally caused by electrical pulses is greatly diminished; examination of a variety of naturally occurring materials for their ability to restore the response showed greatest activity in a fraction of blood-plasma proteins containing α- and β-globulins (Marks & McIlwain, 1959).

Extracts have now been made from cerebral

tissues by a variety of methods, and examined for interaction with the protein fraction (IV-4 of Cohn *et al.* 1946). Certain extracts made with NaCl and ethanol, or with acid, formed precipitates with fraction IV-4. These particular extracts, and not several others, diminished the respiratory response of normal cerebral tissues to electrical pulses. The inhibitory agent did not readily dialyse and was obtained from nuclear but not from other fractions of ground, differentially-centrifuged cerebral tissue. These properties, and the method of extraction, led to the suspicion that the active agent might be a histone.

Histone preparations from calf-thymus nuclei and protamine specimens also inhibited the tissue's response to electrical pulses and to 50 mM-KCl. Plasma fraction IV-4 yielded precipitates with the basic proteins, and restored response to normal cerebral tissues after this had been inhibited by protamines and histones. The basic proteins employed are undoubtedly mixtures, but taking molecular weights of some 4000 and 20 000 they act at about 5 μM in inhibiting the tissue's response; concentrations many times greater were without action on normal respiration or glycolysis. They are thus potent inhibitors showing specificity for action on processes occurring in the excited tissue.

As a working hypothesis, it is suggested that the keeping at 0° may permit migration of histone from the nucleus to acidic sites concerned with the tissue's functional activity, and that from these it can be freed by combination with components of fraction IV-4. The phosphoproteins implicated in utilization of adenosine triphosphate (Heald, 1958), or the polyacidic components postulated in ion transport mechanisms, appear possible targets for the histones.

REFERENCES

Cohn, E. J., Strong, L. E., Hughes, W. L., Mulford, D. J., Ashworth, J. N., Melin, M. & Taylor, H. L. (1946). *J. Amer. chem. Soc.* **68**, 459.

Heald, P. J. (1958). *Biochem. J.* **68**, 580.

Marks, N. & McIlwain, H. (1959). *Biochem. J.* Submitted for publication.

The Cure of the Abnormal Liability to Haemolysis in Vitamin E-Deficient Rats by *NN'*-Diphenyl-*p*-Phenylenediamine. By T. Moore and I. M. Sharman. (*Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council, England*)

Sharman & Moore (1958) have shown that the antioxidant *NN'*-diphenyl-*p*-phenylenediamine (DPPD) can prevent, among other signs of avitaminosis E, the liability to haemolysis by dialuric acid which occurs regularly in the drawn blood of rats deprived of vitamin E (Gyorgy & Rose, 1949). In this former work the antioxidant was given from the time of weaning to animals receiving a diet deficient in vitamin E. The possibility remained open, therefore, that the anti-haemolytic activity was due to the conservation of stores of vitamin E initially present in the animals. Further experiments have therefore been made in which DPPD has been administered to rats which have first been made deficient in vitamin E, by restriction to a basal diet for over 4 months. In two groups of deficient rats, each comprising three males and three females, the percentages of haemolysis found in tests at this point were 79–94 (mean 88) and 77–90 (mean 84). The first group of rats were then kept on the same diet, without supplements, while for the second group the diet was supplemented with 0·125% of DPPD. After a further 26 days haemolysis tests in the first group gave percentages of 72–90 (mean 83), as compared with 0–4·2 (mean 1·1) in the second group. DPPD can therefore correct abnormal haemolysis even in rats which have suffered from prolonged deprivation of vitamin E. This finding supports the view of Draper & Csallany (1958) that DPPD does not owe its vitamin E activity to the protection of the vitamin. It also agrees with a similar conclusion reached by Moore, Sharman & Ward (1955) regarding the partial vitamin E activity of methylene blue, although this dye differs from DPPD in giving no protection against haemolysis.

REFERENCES

Draper, H. H. & Csallany, A. S. (1958). *Proc. Soc. exp. Biol. Med.* **99**, 739.

Gyorgy, P. & Rose, C. S. (1949). *Ann. N.Y. Acad. Sci.* **52**, 231.

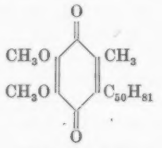
Moore, T., Sharman, I. M. & Ward, R. J. (1955). *Soc. Biol. Chem. India Souvenir*, p. 71.

Sharman, I. M. & Moore, T. (1958). *Biochem. J.* **69**, 61P.

Vitamin A Deficiency and the Ubiquinone and 'SC' of Rat Liver: The Time Factor. By R. A. MORTON and W. E. J. PHILLIPS.† (*Biochemistry Department, University of Liverpool, England*)

Rats suffered loss of appetite in vitamin A deficiency. When they were placed on a vitamin A-deficient diet at weaning, food consumption began to decrease after 14 days in those animals which reached a weight plateau earlier than 27 days after weaning. Some animals took longer to reach a weight plateau and in such cases food consumption continued to increase until about 4 days before growth ceased. Anorexia became marked only in those animals suffering from severe vitamin A deficiency accompanied by declining weight.

Groups of deprived animals were killed after 5, 11, 20 and 28 days while still growing; others were killed at the weight plateau and others at a time of rapid loss of weight. Chromatographic separations (on alumina, Brockmann Grade III) of liver unsaponifiable constituents showed rises, which progressed steadily as the deficiency syndrome developed, in a hydrocarbon with $\lambda_{max.}$ 260 mμ and in ubiquinone 50:



Ubiquinone 50

The livers of rats which were losing weight had on the average 410 μg. of ubiquinone 50/g., i.e. about 5 times as much as the livers of animals killed after 5 days on the deficient diet (82·3 μg./g. of liver, wet weight). The amounts of constituent SC ($\lambda_{max.}$ 230 mμ, 275 mμ, 283 mμ, 332 mμ, $E^{1\%}_{1cm.}$ 217, 96, 91 and 38 respectively) increased after 20 days from 7 to 45 μg./g. Cholesterol also increased after the weight plateau. Male and female animals exhibited similar results throughout.

† Seconded from Chemistry Division, Department of Agriculture, Science Service, Ottawa, Canada.

Osmotic and Chemical Molecular Weights of some Derivatives from the Mucoprotein of Cartilage. By S. M. PARTRIDGE,* H. F. DAVIS and G. S. ADAIR. (*Low Temperature Research Station, Cambridge, England*)

The isolation of the disaccharide chondrosine in high yield from the acid hydrolysis products of chondroitin sulphate (Davidson & Meyer, 1954) indicates the absence of glycosidically linked branch chains. As it exists in cartilage chondroitin sulphate is firmly combined with a protein which is not derived from collagen (Shatton & Schubert, 1954; Partridge & Davis, 1958). The native mucoprotein is of very high molecular weight but this is reduced on destruction of the protein component by the action of proteolytic enzymes (Matthews, 1956). In order to obtain information about the structure of the mucoprotein the polysaccharide chain weight (C), determined by measuring the reducing power due to the terminal residue, has been compared with the molecular weight (M, determined osmotically) for the native complex and various degradation products.

Preparation A (native mucoprotein) was made by extracting powdered bovine nasal cartilage with water at 15–20° using a laboratory homogenizer. The collagen content of the product was reduced to 1–2 % by treatment with an ion exchanger, IRC 50. Found: C, 18 000; M (preliminary value) 140 000 ± 20 000.

Preparation B was made by treating A with papain in the presence of cysteine and versene followed by dialysis through cellophan. M was reduced to 14 000 ± 3000.

Preparation C was made by dissociating the mucoprotein with alkali (Partridge & Davis, 1958) followed by removal of the protein by treatment with IRC 50. The preparation analysed for pure chondroitin sulphate: C, 16 600; M, 16 100.

Preparation D was made from C by partial hydrolysis with testicular hyaluronidase. The reaction was stopped before completion and oligosaccharides of low molecular weight removed by dialysis through cellophan. Found: C, 2150; M, 3800.

The fair measure of agreement between chain weight and osmotic M for preparations C and D establishes the validity of the procedure and shows that non-glycosidic cross-links are absent in the pure polysaccharide. The results obtained with preparations A and B are consistent with the view that the mucoprotein extracted by water is composed of elementary chains of chondroitin sulphate (chain weight about 18 000) linked together by protein to form a molecular unit about ten times this weight.

REFERENCES

Davidson, E. A. & Meyer, K. (1954). *J. Amer. Chem. Soc.* **76**, 5686.
Matthews, M. B. (1956). *Arch. Biochem. Biophys.* **61**, 367.
Partridge, S. M. & Davis, H. F. (1958). *Biochem. J.* **68**, 298.
Shatton, J. & Schubert, M. (1954). *J. biol. Chem.* **211**, 565.

The Action of Lysozyme on the Cell Walls of Gram-Positive Micro-organisms. By H. R. PERKINS.* (*National Institute for Medical Research, Mill Hill, London, N.W. 7, England*)

Lysozyme liberates from the cell walls of certain Gram-positive organisms material giving a reaction for reducing sugar, and also for *N*-acetamido sugar (e.g. Salton, 1956). It has also been reported that material reacting as non-acetylated amino sugar is liberated (Brumfitt, Wardlaw & Park, 1958). In the present work these various reactions have been further examined.

Non-acetylated amino sugar reaction

The result of Ingram & Salton (1957), showing the absence of appreciable numbers of free amino groups on the hexosamines of *Micrococcus lysodeikticus* cell walls treated with lysozyme, has been confirmed. The behaviour of the colour given in the Elson–Morgan test (Rondle & Morgan, 1955) by lysozyme digests of the cell wall was typical of the artifact colour given by mixtures of lysine or glycine and reducing sugar (cf. Immers & Vasseur, 1950). The colour could be much reduced by first converting the ϵ-amino groups of the lysine in the wall digest into either the acetyl or the dinitrophenyl derivatives. Liberation of the reducing group of glucose *per se* was not necessary for this reaction, since the same result could be obtained with cell walls of another organism which contained no hexose. Thus the artifact was probably partly due to the presence in lysozyme digests of free reducing groups on *N*-acetylhexosamine, and free ϵ-amino groups of lysine.

N-Acetamido sugar reaction

The amount of apparent *N*-acetylhexosamine found in the lysozyme digests depended on whether carbonate at pH 10·8 (Aminoff, Morgan & Watkins, 1952) or borate at pH 9·1 (Reissig, Strominger & Leloir, 1955) was used in the test, and also upon the time of heating. The former method gave a maximum apparent *N*-acetylglucosamine content of 2·8% in 12 min., the latter gave a maximum of 1·1% in 3 hr. It was shown chromatographically that free *N*-acetylhexosamine as well as chromogen was liberated under the alkaline reaction conditions (cf. Buchanan, Crumpton & Morgan, 1957). Oligosaccharide material similar to that of Salton (1956) was isolated from lysozyme digests of cell wall. This too gave chromogen slowly in the borate test, and was shown on hydrolysis to contain equimolecular proportions of glucosamine and muramic acid. Examination of the products of hypoiodite oxidation of this material suggested that it was a disaccharide with C-1 of *N*-acetylmuramic acid free. Since chromogen containing unchanged glucosamine could be isolated after alkali treatment, the material was characterized as *N*-acetylglucosaminido(1 → 6)*N*-acetylmuramic acid.

REFERENCES

Aminoff, D., Morgan, W. T. J. & Watkins, W. M. (1952). *Biochem. J.* **51**, 379.
Brumfitt, W., Wardlaw, A. C. & Park, J. T. (1958). *Nature, Lond.*, **181**, 1783.
Buchanan, D. J., Crumpton, M. J. & Morgan, W. T. J. (1957). *Biochem. J.* **65**, 186.
Immers, J. & Vasseur, E. (1950). *Nature, Lond.*, **165**, 898.
Ingram, V. M. & Salton, M. R. J. (1957). *Biochim. biophys. Acta*, **24**, 9.
Reissig, J. L., Strominger, J. L. & Leloir, L. F. (1955). *J. biol. Chem.* **217**, 959.
Rondle, C. J. M. & Morgan, W. T. J. (1955). *Biochem. J.* **61**, 586.
Salton, M. R. J. (1956). *Biochim. biophys. Acta*, **22**, 495.

Gas–Liquid Radiochromatography. By G. POPJÁK,* A. E. LOWE, D. MOORE, L. BROWN and F. A. SMITH. (*Medical Research Council, Experimental Radiopathology Research Unit, Hammersmith Hospital, London, England*)

The construction and performance of a liquid scintillation counter for the measurement of radioactive vapours will be described. The instrument is used in conjunction with a gas–liquid chromatographic instrument for the measurement of ^{14}C contained in chromatographic vapours. With a single photomultiplier tube 50% of the ^{14}C disintegrations are counted and recorded parallel with the chromatographic analysis. The records obtained are of the integral type. The resolving power of the counter is such that radioactive fractions needing only 25 sec. to pass through the chromatographic analyser (gas-density balance) may be clearly distinguished from other radioactive fractions following in 1 min. or less.

The Phospholipids in the Human Aorta. By ELSPETH B. SMITH.* (*Courtauld Institute of Biochemistry, The Middlesex Hospital Medical School, London, W. 1, England*)

As part of an investigation on lipids in relation to atherosclerosis the phospholipids are being studied in the intimal and underlying medial layers of the human aorta, and in different types of atheromatous lesion. Although present in lower concentration than cholesterol, emphasis has been placed on the phospholipids because the distinctive distribution of components gives them an identity which cholesterol does not possess and which may give some indication of the origin of lipids in the arterial wall.

The whole length of the aorta is examined and homogeneous normal or atherosclerotic areas selected for analysis. The intima is stripped off, probably with the internal elastic lamina, and the underlying media is stripped from the outer medial layers and adventitia. The tissue is extracted in cold ethanol/ether, ground to a fine powder and re-extracted with methanol/chloroform. The combined extracts are evaporated under nitrogen, dried *in vacuo*, and dissolved in light petroleum containing 15% of chloroform. The phospholipids are isolated on silicic acid columns (Borgstrom, 1952), and the total non-phospholipid weighed and the cholesterol determined. The phospholipids are separated into lysolecithin, sphingomyelin, lecithin and phosphatydylethanolamine on silica-impregnated papers (Lea, Rhodes & Stoll, 1955); the spots are cut out and eluted, and the phosphorus is determined.

Very marked differences are found between the intimal and underlying medial tissues; in normal areas the intimal fats contain about 35% cholesterol and 20% phospholipid, of which 45–50% is sphingomyelin, whereas the medial fats contain about 40% cholesterol and over 40% phospholipid of which 65–70% is sphingomyelin. This means that comparison of samples containing unknown proportions of intima and media are of doubtful significance, and that it is important that the tissues examined are strictly comparable, particularly when taken from different arteries.

In normal or mildly atheromatous areas the intima can be separated cleanly from the media, but where fat penetrates deeply or the elastic tissue has disintegrated the separation is incomplete. Even allowing for this source of error there is a remarkable increase in the proportion of sphingomyelin with increasing atherosclerosis, and in fully calcified tissue over 80% of the phospholipid is in the sphingomyelin fraction.

Studies on the incorporation of ^{32}P into phospholipids (Zilversmit, 1958; Smith, 1958) indicate that sphingomyelin is metabolized more slowly than other phospholipids, so the increasing proportions of sphingomyelin in older atheromata suggest accumulation rather than an active role in atherogenesis.

REFERENCES

Borgstrom, B. (1952). *Acta physiol. scand.* **25**, 101.
Lea, C. H., Rhodes, D. N. & Stoll, R. D. (1955). *Biochem. J.* **60**, 353.
Smith, E. B. (1958). *4th int. Congress of Biochem.*, Communication 18/28.
Zilversmit, D. B. (1958). *Hormones and Atherosclerosis* (ed. Pincus), p. 145.

The Role of Choline Sulphate in the Sulphur Metabolism of Fungi. By B. SPENCER and T. HARADA. (*Department of Biochemistry, University of Wales, Cardiff, Wales*)

The internal salt choline sulphate,

$$^{-}O_3S\cdot O\cdot CH_2\cdot CH_2\cdot N^{+}(CH_3)_3,$$

has been isolated from the fungi *Aspergillus sydowi* (Woolley & Peterson, 1937) and *Penicillium chrysogenum* (Stevens & Vohra, 1955), lichens of the *Rocella* species (Lindberg, 1955*a*) and from the red alga *Gelida cartilaginum* (Lindberg, 1955*b*). In the fungi more than 0·2% of the weight of the dry mycelium is present as choline sulphate, but although these high concentrations of the sulphate ester would suggest that it is of some importance in the sulphur metabolism of fungi its exact function has not yet been decided. Choline sulphate formation in *A. sydowi* has been shown to involve transfer of sulphate from 3′-phosphateadenosine 5′-sulphate phosphate (PAPS) to choline under the influence of choline sulphokinase (Kaji & McElroy, 1958). The specificity of this transferase has now been studied and will be discussed.

The occurrence of choline sulphate in the mycelium of over thirty fungi grown on media containing radioactive inorganic sulphate has been investigated. All the Moniliales, apart from *Torula utilis*, accumulated choline sulphate in the mycelium but none of the Mucorales and Endomycetales nor any of eight bacteria produced this compound. *Aspergillus oryzae* produced choline sulphate when sulphate, taurine, cysteic acid or cystine were the sole sulphur sources. Experiments *in vitro* using cell-

free extracts have shown the presence of the PAPS-synthesizing system and choline sulphokinase in all the fungi which produce choline sulphate *in vivo*. The failure of certain fungi to accumulate choline sulphate is not due to the rapid utilization of this compound but to the absence of choline sulphokinase. The PAPS-synthesizing system, on the other hand, is present in all fungi which can utilize sulphate as the sole source of sulphur.

A series of *A. nidulans* mutants with blocks in the metabolic pathway between sulphate and cysteine have been examined. The parent strain accumulates choline sulphate when grown on a cysteine-containing medium supplemented with radioactive inorganic sulphate. Under similar conditions some of the mutants could not produce the ester sulphate although all mutants were shown to possess choline sulphokinase. Blocks in the PAPS-synthesizing system were shown to be responsible.

These results, together with studies on the utilization of various sulphur sources in the presence and absence of barium and competitive metabolism experiments using radioactive choline sulphate and inorganic sulphate, suggest that choline sulphate is not an obligatory intermediate in the utilization of sulphate sulphur but that it acts as a store of 'activated sulphate'. The further metabolism of sulphate would seem to be mediated through PAPS formed directly from inorganic sulphate or indirectly by transfer of sulphate from choline sulphate to adenosine 3′:5′-diphosphate (PAP). The next step is probably reductive leading to the formation of sulphite (Wilson & Bandurski, 1958) which is known to be on the metabolic pathway from sulphate to cysteine (Shepherd, 1956).

REFERENCES

Kaji, A. & McElroy, W. D. (1958). *Biochim. Biophys. Acta*, **30**, 190.

Lindberg, B. (1955*a*). *Acta chem. Scand.* **9**, 917.

Lindberg, B. (1955*b*). *Acta chem. Scand.* **9**, 1323.

Shepherd, C. J. (1956). *J. gen. Microbiol.* **15**, 29.

Stevens, C. M. & Vohra, P. (1955). *J. Amer. chem. Soc.* **77**, 4935.

Wilson, L. G. & Bandurski, R. S. (1958). *J. Amer. chem. Soc.* **80**, 5576.

Woolley, D. W. & Peterson, W. H. (1937). *J. biol. Chem.* **124**, 213.

The Distribution of Polysaccharides in Hyaline Cartilage. By J. A. Szirmai and J. Doyle.
(*Research Laboratories, Department of Rheumatology, University Hospital, Leiden, The Netherlands*)

Chondroitin sulphates A and C and keratosulphate are considered to be major constituents of the cartilage ground substance (Meyer, Hoffman & Linker, 1958). In addition, neutral sugar components have also been described (Stidworthy, Masters & Shetler, 1958). The composition of cartilage has been shown by these authors to vary with age, with keratosulphate appearing more in old cartilage.

On the basis of the growth pattern of cartilage, one might expect to find age differences reflected locally in the various layers of this tissue. Local differences between the staining properties of various layers of hyaline cartilage have been observed using azure A at different pH values. There is a close connexion between the metachromatic staining and the dye binding determined quantitatively (Szirmai & Balazs, 1958). Since the dye binding is a quantitative measure of the free anionic sites, a detailed study of it has been made in cartilage.

Serial sections of cartilage from horse nasal septum were cut parallel to the surface in a cryostat at −15°. The wet weight of the sections was determined while still frozen, on an electrobalance, and alternate groups of sections used to determine dye binding at pH 2 and 7, and for microscopic study of the metachromasia at these two pH values. 0·2 mM Azure A in water or HCl was used, and the results expressed as micromoles of dye bound per 100 mg. of tissue. From calculations of average dye binding three distinct zones appeared, related to the structure of the tissue observed histologically. An attempt has been made to explain the staining behaviour and dye binding of these three layers in terms of their relation to chemical composition as studied on layers isolated by dissection.

REFERENCES

Meyer, K., Hoffman, P. & Linker, A. (1958). *Science*, **128**, 896.

Stidworthy, G., Masters, Y. F. & Shetler, M. R. (1958). *J. Gerontol.* **13**, 10.

Szirmai, J. A. & Balazs, E. A. (1958). *Acta Histochem* Suppl. 1, p. 56.

The Purification and Properties of Thyroxine Deiodinase from Skeletal Muscle. By J. R. TATA.*†
(*National Institute for Medical Research, Mill Hill, London, N.W. 7, England*)

Enzymic deiodination of iodotyrosines, iodothyronines and their deaminated analogues *in vitro* has been recently described. With the exception of skeletal muscle, deiodination of thyroxine and tri-iodothyronine with non-thyroid tissues is accompanied by other reactions (Tata, 1957). Rabbit skeletal muscle was therefore selected for experiments on the extrathyroidal deiodinating enzyme.

The following procedures were adopted for a partial purification of the enzyme: (*a*) reduction in ionic strength of the soluble fraction of muscle homogenate, (*b*) heat treatment, (*c*) ammonium sulphate fractionation and (*d*) adsorption on and elution from calcium phosphate gel. The properties of the resulting enriched material were studied.

Enzyme activity was measured by paper chromatographic analysis using ^{131}I-labelled L-thyroxine or tri-iodo-L-thyronine as substrates. Formation of radioactive iodide (or its incorporation into proteins) was used as a measure of deiodination. True enzymic deiodination was distinguished from an apparent reaction by a recently-described test (Tata, 1959).

The enzyme was unstable after storage, lyophilization and freezing and thawing. It was sensitive to the usual sulphydryl group poisons. Fe^{2+} ions and flavins (riboflavin, flavin mononucleotide and flavinadenine dinucleotide) were found to be the only activators among a large group of substances tested. Consequently deiodination was inhibited by chelators of divalent metal ions and flavin antagonists. Partial reactivation of the $HgCl_2$-inactivated enzyme by the addition of flavin mononucleotide was demonstrable; a similar effect has been observed for D-amino acid oxidase (Frisell & Hellerman, 1957).

Studies on substrate specificity indicated the affinity of the deiodinase in the following decreasing order: thyroxine, tri-iodothyronine, tetraiodothyroacetic acid = tri-iodothyroacetic acid, di-iodotyrosine = monoiodotyrosine. The enzyme could also dehalogenate bromothyronines and bromotyrosines. In general, the affinity of the enzyme for the above substances was comparable to that for serum thyroxine-binding protein which was found to be a powerful inhibitor of the deiodination of thyroxine and tri-iodothyronine. Only deiodination of 'free' or non-protein-bound hormone could be catalysed by the enzyme. The major iodinated product was iodide but 10–25% of ^{131}I was consistently found to be incorporated into proteins. Tri-iodothyronine was not found as a product of deiodination of thyroxine.

The substrate specificity of thyroxine deiodinase in skeletal muscle and its activation by Fe^{2+} ions and flavin prove that the enzyme is different from the triphosphopyridine nucleotide-dependent deiodinase described by Stanbury (1957).

† Beit Memorial Fellow.

REFERENCES

Frisell, W. R. & Hellerman, L. (1957). *J. biol. Chem.* **225**, 53.
Stanbury, J. B. (1957). *J. biol. Chem.* **228**, 801.
Tata, J. R. (1957). *Proc. Soc. exp. Biol., N.Y.*, **95**, 362.
Tata, J. R. (1959). *Biochem. J.* **72**, 222.

The Specificity of Elastases. By J. THOMAS*† and S. M. PARTRIDGE.* (*Low Temperature Research Station, Cambridge, England*)

A number of known proteolytic and mucolytic enzymes have been assayed for elastolytic activity using as substrate a preparation of elastin of known purity. This contained less than 0·2% of carbohydrate and was free from collagen.

Excluding pancreatic elastase, three proteolytic enzymes papain, ficin and bromelin, were found to have a high activity. Crystalline trypsin, chymotrypsin, pepsin, 'Rhozyme', and 'kathepsin' from kidney and spleen were found to be inactive. Four preparations of α-amylase from different sources were assayed. The preparations from saliva and cereal seeds were inactive but a crystalline sample of pancreatic α-amylase showed a low activity. However, this activity disappeared when the preparation was recrystallized and it was concluded that the elastolytic activity of α-amylase reported in the literature is due to contamination with pancreatic elastase. Preparations of bacterial chondrosulphatase and purified chondroitinase also showed no activity.

Various commercial preparations of ficin all showed the same ratio of elastolytic to proteolytic activity, the latter being measured by following the

† Empire Rheumatism Council Fellow.

reduction in viscosity of a gelatin solution. This same ratio was maintained throughout a careful fractionation and purification of commercial dried-fig latex. It was therefore concluded that 'fig latex elastase' is identical with the major proteolytic enzyme in preparations of 'ficin'.

The results of the survey confirm the conclusion of Partridge & Davis (1955) that elastolysis is the result of proteolytic activity. The failure of many well characterized proteolytic enzymes such as trypsin and chymotrypsin to dissolve elastin may be attributed either to their failure to penetrate the cross-linked structure of elastin or to the very narrow peptide bond specificity exhibited by these enzymes.

REFERENCE

Partridge, S. M. & Davis, H. F. (1955). *Biochem. J.* **61**, 21.

The Subcellular Localization of Transmitter Substances in the Central Nervous System. By V. P. WHITTAKER.* (*Agricultural Research Council, Institute of Animal Physiology, Babraham, England*)

Synaptic transmission is being currently discussed in terms of the synaptic vesicle theory (see Eccles, 1957). Synaptic vesicles are small bodies 0·02–0·08 μ in diameter visible in electron micrographs of nerve endings (for three-dimensional reconstructions and a review of earlier work see Andersson-Cedergren, 1959). They are assumed to contain transmitter substances and to discharge on the arrival of a nerve impulse at the nerve ending. Their spontaneous breakdown at a low rate could account for the 'miniature end-plate potentials' observed by Castillo & Katz (1958) in resting end-plates, and their physical and chemical properties, for the phenomenon of 'bound' transmitter substances.

Arising from studies of the subcellular distribution of choline acetylase in nervous tissue (Hebb & Smallmann, 1956) and the discovery that this enzyme existed in sucrose homogenates of brain in an 'occluded' or 'sequestered' form from which it must be liberated for full activity, Hebb & Whittaker (1958) showed that bound acetylcholine and choline acetylase were similarly distributed and were associated with a fraction of subcellular particles distinct from mitochondria. Further studies (Whittaker, 1958, 1959*a*, *b*) have shown that this fraction consists largely of simple vesicles 0·02–0·3 μ in diameter (median, 0·065 μ) and also contains a high proportion of the bound 5-hydroxytryptamine of the tissue. It is thus likely, on the chemical and morphological evidence, that the preparation consists mainly of a mixed population of synaptic vesicles from different types of nerve endings and may be a rich source of other as yet unidentified transmitter substances. Part of the acetylcholine and 5-hydroxytryptamine is bound in a highly labile state and can be readily released by mildly disruptive procedures—freezing and thawing, hypotonic dilution, mechanical agitation, treatment with cobra venom lecithinase—such as are known to increase the permeability of subcellular particles. That the acetylcholine and 5-hydroxytryptamine containing particles are not identical is shown by the different distribution of the two substances in brain and the selective depletion of 5-hydroxytryptamine by reserpine.

The biochemical and pharmacological properties of the particles are being studied in an attempt to throw light on the mechanism of release of transmitter substances *in vivo*.

REFERENCES

Andersson-Cedergren, E. (1959). *J. Ultrastructure Res.* Suppl. 1.
Castillo, J. del & Katz, B. (1958). *Progr. Biophys.* **6**, 121.
Eccles, J. C. (1957). *The Physiology of Nerve Cells.* Baltimore: The Johns Hopkins Press.
Hebb, C. O. & Smallmann, B. (1956). *J. Physiol.* **134**, 385.
Hebb, C. O. & Whittaker, V. P. (1958). *J. Physiol.* **142**, 187.
Whittaker, V. P. (1958). *Abstr. IV int. Congr. Biochem.* p. 74.
Whittaker, V. P. (1959*a*). *Biochem. Pharmacol.* **1**, 351.
Whittaker, V. P. (1959*b*). *Biochem. J.* (in the Press).

[*The Editors of the Biochemical Journal accept no responsibility for the Reports of the Proceedings of the Society.*]

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **387th Meeting** *of the Society was held in the Department of Biological Chemistry, University of Aberdeen on Friday,* 18 *September, when the following papers were read:*

COMMUNICATIONS

Effect of Insulin on the Incorporation of ^{14}C from ^{14}C-Labelled Carboxylic Acids and Bicarbonate into the Protein of Isolated Rat Diaphragm. By K. L. MANCHESTER* and M. E. KRAHL. (*Department of Physiology, University of Chicago, U.S.A.*)

Insulin *in vitro* can promote incorporation of [^{14}C]-amino acids into protein of isolated rat diaphragm (Krahl, 1952, 1953; Sinex, MacMullen & Hastings, 1952), by an action that appears not to depend on the transfer of glucose from the extracellular to intracellular fluid of the muscle cell (Manchester & Young, 1958; Krahl & Wool, 1958; Wool & Krahl, 1959*a*). The question has been raised (Kipnis & Noall, 1958) as to whether this increased incorporation of [^{14}C]amino acid into protein of diaphragm may result from a stimulation by insulin of the transfer of labelled amino acid from extracellular into intracellular fluid. The present experiments, however, indicate that insulin enhances the incorporation of amino acids into the protein of diaphragm by a mechanism which is independent of a stimulation of the transport of amino acid from the medium into the cell.

Hemi-diaphragms from non-fasting female Sprague–Dawley rats (100–120 g. wt.) were incubated for 2 hr. at 37° in Krebs–bicarbonate medium, containing no added glucose, with or without insulin (0·1 unit/ml.), in the presence of $NaH^{14}CO_3$, [^{14}C]formate, [1-^{14}C]acetate, [1-^{14}C]-propionate, [3-^{14}C]pyruvate, [1-^{14}C]*iso*butyrate, [2-^{14}C]succinate, [5-^{14}C]oxoglutarate or [1,5-^{14}C]-citrate. The protein was isolated as previously described (Wool & Krahl, 1959*a*). In every case insulin produced a highly significant stimulation of incorporation of radioactivity into the protein fraction, the percentage increase ranging from 25% for citrate to 84% for *iso*butyrate. In the majority of cases addition of insulin produced no increase in the quantity of soluble radioactive material (assumed to be mainly carboxylic acid) in the tissue during incubation.

It is concluded that insulin enhances the incorporation into protein of amino acids formed from various precursors within the cell and that this stimulating action of insulin is not due to an effect of insulin on the entry of glucose, amino acids or other metabolites into the cell. These results are in accord with the observation of Wool & Krahl (1959*b*) that insulin also stimulates the incorporation into diaphragm protein of labelled amino acids accumulated in the tissue before insulin is added.

This work has been aided by grants from the National Institutes of Health and Eli Lilly and Company. One of us (K.L.M.) is grateful to the Medical Research Council for a personal grant.

* Permanent address: Department of Biochemistry, University of Cambridge.

REFERENCES

Kipnis, D. M. & Noall, M. W. (1958). *Biochim. biophys. Acta*, **28**, 226.
Krahl, M. E. (1952). *Science*, **116**, 524.
Krahl, M. E. (1953). *J. biol. Chem.* **200**, 99.
Krahl, M. E. & Wool, I. G. (1958). *IVth int. Biochem. Congr., Vienna*, 1–6 September, Abstracts 9–4.
Manchester, K. L. & Young, F. G. (1958). *Biochem. J.* **70**, 353.
Sinex, F. M., MacMullen, J. & Hastings, A. B. (1952). *J. biol. Chem.* **198**, 615.
Wool, I. G. & Krahl, M. E. (1959*a*). *Amer. J. Physiol.* **196**, 961.
Wool, I. G. & Krahl, M. E. (1959*b*). *Nature, Lond.*, **183**, 1399.

The Enzymic Formation of Haem by Human and Rat Tissues. By ANN C. LOCHHEAD and A. GOLDBERG (introduced by C. RIMINGTON). (*University Department of Medicine, Gardiner Institute, Western Infirmary, Glasgow, W.* 1)

Chicken erythrocytes or human reticulocytes contain an enzyme system which incorporates iron into protoporphyrin to form haem (Goldberg, 1959). Ascorbic acid or reduced glutathione (GSH) when added to the enzyme system derived from these sources causes a marked potentiation of iron incorporation. The present studies have shown that homogenates of human and rat liver, spleen and human bone marrow, are also sources of the iron-incorporating enzyme system (I.I.E.).

The addition of ascorbic acid and GSH caused some potentiation of the iron-incorporating enzyme

activity of all the tissues. Dialysis of the tissue homogenates against water led to a marked loss of activity as well as a considerable reduction in their contents of ascorbic acid and GSH. The enzymic activity was restored on addition of ascorbic acid or GSH to the dialysed extract.

The intracellular components of human and rat liver were isolated by the method of Schneider (1948). Of these components the mitochondria were the richest source of I.I.E. This evidence is a link with the work of Bessis & Breton-Gorius (1957) who showed that ferritin iron is mainly concentrated in the mitochondria of the haemoglobin-forming erythroblast. The enzyme activity of the mitochondria was also potentiated by the addition of ascorbic acid and GSH.

These studies have therefore confirmed the importance of ascorbic acid and GSH in the mechanism of iron incorporation.

REFERENCES

Bessis, M. & Breton-Gorius, J. (1957). *C.R. Acad. Sci., Paris*, **244**, 2846; **245**, 1271.
Goldberg, A. (1959). *Brit. J. Haemat.* **5**, 150.
Schneider, W. C. (1948). *J. biol. Chem.* **176**, 259.

The Specificity of Preparations with *N*-Acetyl-β-glucosaminidase and *N*-Acetyl-β-galactosaminidase Activities. By J. W. WOOLLEN, R. HEYWORTH and P. G. WALKER. (*Department of Biochemistry, The Institute of Orthopaedics, Royal National Orthopaedic Hospital, Stanmore, Middlesex*)

The specificity of *N*-acetyl-β-D-glucosaminidase from three sources has been studied using phenyl and *p*-nitrophenyl *N*-acetyl-β-D-glucosaminides (Leaback & Walker, 1957) and the corresponding galactosaminides (Heyworth & Leaback, 1958) as substrates.

Competition has been demonstrated between glucosaminide and galactosaminide with extracts of sweet almond meal (Heyworth, Borooah & Leaback, 1957), but ammonium sulphate fractionation and heat inactivation produced changes in the ratio of the two activities indicating that two or more enzyme proteins of unknown specificity are present.

However with extracts of rat kidney (Pugh, Leaback & Walker, 1957) and ram testis no separation of the two activities has been achieved by either of these methods or by calcium phosphate gel absorption. K_i values have been measured against the respective *p*-nitrophenyl substrates for the competitive inhibitors *N*-acetylglucosamine, 2-acetamido-2-deoxygluconolactone, phenyl *N*-acetylglucosaminide and the corresponding galactosamine derivatives. Using the *N*-acetylhexosamines no evidence of *partially* competitive inhibition was obtained.

With the kidney preparation differences in the respective K_i values suggested the presence of two active sites. This was supported by independent estimations of each liberated aglycone from mixtures of phenyl *N*-acetylglucosaminide and *p*-nitrophenyl *N*-acetylgalactosaminide and by calculations from separately determined $V_{max.}$, K_m and K_i values for two specifically active sites, each being competitively inhibited by the other substrate (cf. Friedenwald, Gryder & Gryder, 1958). Apparently these two sites exist on one enzyme protein.

With the testicular preparation the similarity of the respective K_i values suggested the existence of only one enzyme site. This was supported by the agreement of the amount of hydrolysis in mixtures of glucosaminide and galactosaminide substrates and the amount calculated from individual K_m and $V_{max.}$ values for competition at one site (Foster & Niemann, 1951). The alternative possibility of two specific sites with substrates giving mutual competitive inhibition *and* with K_m equal to K_i for each substrate and in this case, the respective K_i values of the other inhibitors being equal, seems highly improbable.

We are indebted to Dr G. A. Levvy for a gift of 2-acetamido-2-deoxygalactonolactone, and to Messrs Bengers Ltd. for the freeze-dried testis extract.

REFERENCES

Foster, R. J. & Niemann, C. (1951). *J. Amer. chem. Soc.* **73**, 1552.
Friedenwald, J. S., Gryder, R. M. & Gryder, J. W. (1958). *Arch. Biochem. Biophys.* **73**, 262.
Heyworth, R., Borooah, J. & Leaback, D. H. (1957). *Biochem. J.* **67**, 21P.
Heyworth, R. & Leaback, D. H. (1958). *Chem. & Ind.* p. 1145.
Leaback, D. H. & Walker, P. G. (1957). *J. chem. Soc.* p. 4754.
Pugh, D., Leaback, D. H. & Walker, P. G. (1957). *Biochem. J.* **65**, 464.

Investigation of the Unreacted Fluorescent Material in Fluorescein–Protein Conjugates. By C. S. CHADWICK, R. C. NAIRN and M. G. MCENTEGART. (*Departments of Biochemistry, Pathology and Bacteriology, University of Aberdeen*)

Fluorescent dyes can be combined with immune sera without material loss of antibody potency; the resulting conjugates may be used as specific immunohistochemical stains. This fluorescent tracing technique was developed by Coons and his colleagues (1942, 1956) who used the *iso*cyanate linkage for coupling fluorescein to protein. After reaction at pH 9, the conjugate is dialysed to remove unreacted fluorescent material. For use as a stain the dialysed conjugate is treated with acetone-dried tissue powders to reduce non-specific staining of tissue sections. Non-specific staining is partly attributable to competition by the tissue section for unreacted fluorescent material adsorbed on the conjugate and not removed by dialysis.

Repeated absorptions of conjugates with tissue powders removes only 50–75% of the unreacted fluorescent material; the rest remains adsorbed on the conjugate and may be removed almost completely with powdered activated charcoal. The following experiment demonstrates this property of charcoal. Fluorescein *iso*cyanate stirred overnight with buffered saline (pH 9) yielded a fluorescent solution which was then mixed with serum. The product remained brightly fluorescent after dialysis and two absorptions with tissue powder, but fluorescence was almost completely abolished by shaking for 1 hr. with activated charcoal. Fluorimetric, absorptiometric and protein determinations, carried out on a crystalline bovine albumin conjugate, indicated that 65% of the adsorbed fluorescent material was removed by two tissue powder absorptions and a further 33% by charcoal absorption. A similar experiment using fluorescein *iso*thiocyanate for conjugation gave much the same results.

The staining power of conjugated antisera treated with tissue powder only was compared with that of the same conjugates treated with charcoal. The latter conjugates possessed about two-thirds the staining power of the former but stained more specifically; the adsorbed dye evidently contributes to staining potency of conjugates. This is not necessarily detrimental to the method provided specificity of staining is properly controlled. However, the amount of adsorbed dye in fluorescein conjugates is inconstant with the practical implication that there is uncertainty about the proportion of chemically combined dye present. For quantitative biological experiments, such as the determination of plasma protein distribution and clearance, fluorescein-labelled proteins should be absorbed with charcoal.

The nature of the fluorescent material adsorbed on the conjugated protein is under investigation.

REFERENCES

Coons, A. H. (1956). *Int. Rev. Cytol.* **5**, 1,
Coons, A. H., Creech, H. J., Jones, R. N. & Berliner, E. (1942). *J. Immunol.* **45**, 159.

Aconitase Levels in the Leaves of Iron-deficient Mustard Plants. By J. S. D. BACON, P. C. DEKOCK and M. J. PALMER. (*Macaulay Institute, Aberdeen*)

In view of the association of iron with the activity of aconitase from animal tissues (Dickman & Cloutier, 1950; Morrison, 1954) it was thought possible that iron deficiency would reduce the level of aconitase in plants. The method of Racker (1950) has been adapted to measure aconitase activity in leaf homogenates made with 0·5M-sucrose, 0·2M-tris buffer (pH 8·5) and 0·005M-EDTA.

Homogenates were centrifuged for 3–5 min. at 1000 *g* to remove cell debris, chloroplasts, etc., and small samples (0·025–0·10 ml.) added to two 1 cm. spectrophotometer cells. Each cell contained (in a total of 3·0 ml.) 0·1M-tris buffer (pH 8·0) and 0·5M-sucrose; the test cell contained in addition 0·05M-DL-*iso*citrate. The increase in optical density at 240 mμ was followed for 30 min.; it was usually linear, and the slope was proportional to the volume of sample taken. The aconitase activity calculated from the change in O.D. was as high as 200 μmoles/hr./g. fresh weight with leaves of some species (e.g. *Sambucus nigra*) and negligible with others (*Coleus blumei*).

Mustard (*Sinapis alba*) was grown in nutrient solutions (DeKock, 1956) containing 0·1, 0·5 and 2·5 p.p.m. ferric EDTA. The leaves of those plants receiving the least iron showed severe chlorosis and always contained substantially less aconitase activity (9 μmoles/hr./g.) than those from the 0·5 p.p.m. plants (19 μmoles/hr./g.). Differences in aconitase level between the 0·5 and 2·5 p.p.m. treatments seemed to depend upon the leaf selected for analysis, and the results were consistent with the idea that the early (1st and 2nd) leaves of the

2·5 p.p.m. plant age more quickly and lose their aconitase activity. Later leaves (5th, 6th, 7th) had at least as much aconitase as those from the 0·5 p.p.m. plants, sometimes more (up to 44 μmoles/hr./g.).

The aconitase activity of the homogenates from 0·1 p.p.m. plants was not increased by incubation at 0° with ferrous ions and cysteine (cf. Morrison, 1954).

Statistical analysis showed no effect of potassium or phosphorus levels on the leaf aconitase. Measurements of malic dehydrogenase activity by a modification of the method of Price & Thimann (1954) showed only a small effect of iron level, suggesting that the lower aconitase activity is a specific effect of iron deficiency.

This work was done with the technical assistance of Miss D. M. Stewart, and thanks are due to Mr R. H. E. Inkson for the statistical analyses.

REFERENCES

DeKock, P. C. (1956). *Ann. Bot., Lond.*, **20**, 133.
Dickman, S. R. & Cloutier, A. A. (1950). *Arch. Biochem. Biophys.* **25**, 229.
Morrison, J. F. (1954). *Biochem. J.* **56**, 99.
Price, C. A. & Thimann, K. V. (1954). *Plant Physiol.* **29**, 113.
Racker, E. (1950). *Biochim. biophys. Acta*, **4**, 211.

L-α-Glycerophosphate Oxidase and the Terminal Respiratory Pathway of the Bloodstream Form of *Trypanosoma rhodesiense*. By P. T. Grant and J. R. Sargent. (*Department of Biological Chemistry, University of Aberdeen*)

Water homogenates of the African trypanosome, *Trypanosoma rhodesiense*, yield an optically clear supernatant on centrifugation at 1000 *g* for 10 min. This supernatant contains a very active DPN-linked L-α-glycerophosphate (L-α-GP) dehydrogenase, similar to that found in mammalian muscle. The equilibrium constant of the reaction (1) greatly favours the formation of L-α-GP from dihydroxyacetone phosphate (DHAP).

The washed residue resuspended in phosphate buffer pH 7·4 contain an oxidase system specific for L-α-GP catalysing reaction (2). Negligible oxygen uptakes were observed when equimolar amounts of DPNH, DPN^+, succinate, lactate, malate, *iso*-citrate, glycerol or β-glycerophosphate were used as substrates. This oxidase, like the intact trypanosome, does not contain detectable cytochrome pigments and is moreover insensitive to cyanide, azide, antimycin A, *n*-heptyl hydroxyquinoline *N*-oxide or EDTA. In these respects it differs from the cytochrome-linked L-α-GP oxidase present in insect flight-muscle mitochondria (Estabrook & Sacktor, 1958).

The oxygen consumption of intact trypanosomes has been directly compared with that of the crude oxidase obtained from the same number of parasites. In a typical experiment lasting 30 min., a constant rate of 11·6 μl. O_2/min./mg. N was found for the intact parasite suspended in phosphate buffer containing glucose (3 μM/ml.), while the oxidase in the same buffer containing L-α-GP (6 μM/ml.) had a rate of 8·2 μl. O_2/min./mg. N of trypanosome used to obtain the preparation.

It has been shown previously that *T. rhodesiense* suspended in saline catabolizes added glucose by the Embden–Meyerhof–glycolytic pathway, the products being pyruvate with a trace of glycerol (Grant & Fulton, 1957). Under these conditions approximately one mole of oxygen is taken up per mole of glucose utilized, so that a system for the re-oxidation of DPNH formed in the triose phosphate dehydrogenase reaction must be present. In view of the low direct rate of DPNH oxidation observed, it is suggested that the reactions catalysed by the soluble L-α-GP dehydrogenase (1) and the particulate oxidase (2) are coupled (see, Estabrook & Sacktor, 1958) and provide a hydrogen carrier system for the re-oxidation of DPNH.

$$\text{DHAP} + \text{DPNH} + \text{H}^+ \rightleftharpoons \text{L-α-GP} + \text{DPN}^+ \quad (1)$$

$$\text{L-α-GP} + \tfrac{1}{2}\text{O}_2 \rightarrow \text{DHAP} + \text{H}_2\text{O} \quad (2)$$

$$\text{sum.} \quad \text{DPNH} + \text{H}^+ + \tfrac{1}{2}\text{O}_2 \rightarrow \text{DPN}^+ + \text{H}_2\text{O}$$

REFERENCES

Estabrook, R. W. & Sacktor, B. (1958). *J. biol. Chem.* **233**, 1014.
Grant, P. T. & Fulton, J. D. (1957). *Biochem. J.* **66**, 242.

An Inhibitory Effect of Mepacrine on the Catabolism of Glucose by *Plasmodium berghei*. By I. B. R. Bowman, P. T. Grant, W. O. Kermack and D. Ogston. (*Department of Biological Chemistry, University of Aberdeen*)

Plasmodium berghei, the malaria parasite of rodents, was separated from the host reticulocyte by differential lysis (Bowman, Cook, Grant & Kermack, 1957) and the pathways of glucose catabolism in this free parasite were studied by the use of [1-^{14}C]- and [6-^{14}C]-glucose. The distribution of ^{14}C in the lactate produced and the low yield of $^{14}CO_2$ was consistent only with the Embden–Meyerhof-

glycolytic pathway. A similar result was also obtained for the simian parasite, *P. knowlesi*, which had been previously separated from monkey red cells by saponin haemolysis.

The major products of catabolism of [U-^{14}C]-glucose were organic acids and hexose-6-phosphate which accounted for about 82 and 8 % respectively of the glucose utilized. The acid fraction was essentially all lactate. Minor amounts of radioactivity were detected in the parasite material and respiratory CO_2. Mepacrine (10^{-5} M) caused a marked reduction in the utilization of [U-^{14}C]-glucose by the free parasite. Under these conditions the proportion of the glucose utilized which could be accounted for as the organic acid fraction decreased to 64 % while that of the hexose-6-phosphates increased to 31·5 %. In both the normal and drug-treated suspensions, hexose-6-phosphate which accumulated was glucose-6-phosphate together with smaller amounts of fructose-6-phosphate. These results suggest that either phosphohexosisomerase or phosphofructokinase is a rate-limiting step in glycolysis by the free parasite and that mepacrine has a marked inhibitory action on either or both of these enzymes.

Mepacrine also markedly decreased the glucose utilization of whole infected reticulocytes although it was without effect on normal reticulocytes. The yields of $^{14}CO_2$ from [U-^{14}C]glucose were less than 3 % of the glucose utilized by normal or infected red cells, and were not significantly increased by mepacrine. These results suggest that the inhibitory effect on the metabolism of the free parasite may also be a selective effect of mepacrine on the parasite in its natural environment, the reticulocyte.

REFERENCE

Bowman, I. B. R., Cook, Linda R., Grant, P. T. & Kermack, W. O. (1957). *Biochem. J.* **67**, 23P.

Human Adipose Tissue. By C. H. Moore and R. P. Cook. (*Biochemistry Department, Queen's College, Dundee, University of St Andrews, Scotland*)

Samples of human adipose tissue (subcutaneous, omental, epicardial and perinephric) extracted with ethyl ether (1 vol.)–acetone (1 vol.) yielded (as g./100 g. fresh tissue) connective tissue 1–2, 'lipid' 69–85, water 7–30. The lipid extract chromatographed on silicic acid–celite with benzene yielded fraction (i) mainly glyceride with a small amount of steryl ester, 90–97 %; with benzene (9 vol.)–ethyl ether (1 vol.) fraction (ii) glycerides and free sterol, 1–13 %; with ethyl ether (9 vol.)–methanol (1 vol.), a small unidentified fraction. The benzene fraction after methanolysis (Rowe, 1959) was studied by gas–liquid chromatography (Martin & James, 1956) using polyethyleneglycol adipate as stationary phase (Lipsky & Landowne, 1958). The fatty acid composition (as % total acids) showed oleic 42–51, palmitic 22–31, palmitoleic 5–14, stearic 2–15, myristic 3–5, linoleic 1–6, lauric 1–2, small amounts of C_{15} and C_{17} acids were present. Subcutaneous fat has generally the highest iodine value apparently dependent on the relative amounts of oleic, palmitoleic and linoleic acids. The analyses are in general agreement with those of Cramer & Brown (1943).

We are indebted to Mr W. G. Campbell and Mr J. G. S. Blair of Maryfield Hospital, Dundee, for the fresh samples of tissue and to the Scottish Hospital Endowments Research Trust for a grant.

REFERENCES

Cramer, D. L. & Brown, J. B. (1943). *J. biol. Chem.* **151**, 427.
Lipsky, S. R. & Landowne, R. A. (1958). *Biochim. biophys. Acta*, **27**, 666.
Martin, A. J. P. & James, A. T. (1956). *Biochem. J.* **63**, 138.
Rowe, C. E. (1959). Personal communication.

Action of Inhibitors on an Amino Acid Decarboxylase: Nature of the Enzyme Centre. By C. R. Sutton and H. K. King. (*Department of Biochemistry, The University of Liverpool*)

Proteus vulgaris possesses an enzyme which decarboxylates certain neutral amino acids (Ekladius, King & Sutton, 1957). It requires pyridoxal phosphate (PLP) as coenzyme and is completely inhibited by equimolar hydroxylamine, PLP-oxime, or PLP-cyanhydrin. Excess coenzyme will reverse these inhibitions.

It is also inhibited by mercury and silver salts (reversed by thiols or hydrogen sulphide) and by 10^{-5} M *p*-chlormercuribenzoate (PCMB) and 10^{-3} M-iodoacetate. Iodoacetate inhibition is irreversible, but PLP can protect the enzyme if added before, or simultaneously with, the inhibitor. Protection is related to PLP concentration in a manner

suggestive of competitive inhibition. Substrates of the enzyme accentuate iodoacetate inhibition. This is notably the case with leucine; significantly, enzyme–coenzyme affinity is known to be lower when this substrate is being attacked. *iso*Butylamine, *iso*valeraldehyde, and *iso*valeric acid inhibit the enzyme by competition with the substrate. The first two of these do not influence iodoacetate inhibition: *iso*valeric acid protects the enzyme slightly.

Analogues of the coenzyme lacking the –CHO group (PLP-oxime and PLP-cyanhydrin, and pyridoxamine phosphate) fail to protect the enzyme against iodoacetate inhibition. It is suggested that iodoacetate attacks an –SH group which is normally bound to and protected by the –CHO group of the coenzyme; when the latter becomes involved in Schiff base formation with the substrate this –SH group is exposed to attack by iodoacetate. (Cf. Matsuo & Greenberg, 1959; Jenkins & Sizer, 1957.)

By contrast, PCMB inhibition is reversible—indeed, gradual spontaneous recovery occurs as substrate decarboxylation proceeds. The coenzyme does not markedly influence inhibition but considerable *protection* is afforded by the substrates, though *iso*valeric acid has no action. The PCMB-sensitive group is thus not apparently involved in binding the substrate –COOH, nor, presumably, the substrate $-NH_2$ since the latter is believed to be bound to the coenzyme –CHO group. Iodoacetate evidently attacks a different group to PCMB; the group attacked by the latter may fulfil a 'catalytic' rather than a substrate-binding function. Indeed, the protection afforded by the substrate suggests that the PCMB-sensitive group may come into close contact with it during enzyme action.

REFERENCES

Ekladius, L., King, H. K. & Sutton, C. R. (1957). *J. gen. Microbiol.* **17**, 602.

Jenkins, W. T. & Sizer, I. W. (1957). *J. Amer. chem. Soc.* **79**, 2655.

Matsuo, Y. & Greenberg, D. M. (1959). *J. biol. Chem.* **234**, 507.

Carbohydrate Fermentation by Rumen Oligotrich Protozoa of the Genus *Entodinium*.

By A. R. Abou Akkada, P. N. Hobson and B. H. Howard. (*The Rowett Research Institute, Bucksburn, Aberdeen*)

Biochemical investigations of holotrich protozoa (Sugden & Oxford, 1952; Heald & Oxford, 1953; Gutierrez, 1955; Howard, 1959*a*, *b*) have indicated the part played by these organisms in ruminal carbohydrate fermentation. The difficulty of obtaining pure, active suspensions has hindered similar experiments with the rumen oligotrichs.

Rumen liquid, withdrawn 3 hr. after the concentrate feed from a sheep, partially defaunated (Eadie & Oxford, 1957) and fed on hay (7 a.m., 4 p.m.) and concentrates (11 a.m.), yielded a clean active suspension of entodinia when treated according to the method of Oxford (1958). The suspensions were freed from holotrichs by incubation with mannose, and bacteria were suppressed by chloramphenicol (Coleman, 1958). The predominant organisms present were similar to *Entodinium caudatum*; *E. simplex* and *E. longinucleatum* were present in smaller numbers. The protein and starch contents of the cells were initially 24 and 64% respectively, of the dry matter. Incubation of the suspensions was anaerobic, and carried out in Coleman's (1958) buffer, except that acetate was replaced by bicarbonate when volatile fatty acid (VFA) was to be determined.

In the absence of external substrate, cells filled with plant starch from the concentrate feed metabolized 60 and 91% of this respectively during incubation for 24 and 48 hr. Similarly, the rates of endogenous fermentation in a bicarbonate buffer, measured manometrically under an atmosphere of N_2/CO_2, were 38, 21 and 16 μl. CO_2/0·1 mg. cell N/hr. respectively for suspensions, 5, 24 and 48 hr. old. When granular rice starch was added to a 36 hr. old suspension the fermentation rate 12 hr. later had risen to 22 μl. CO_2/0·1 mg. cell N/hr. The end products of endogenous starch digestion were lactic acid, VFA, CO_2 and 'other gas', in a molar ratio of 1, 44, 30 and 26 respectively. VFA were butyric, propionic, acetic and formic acids in the molar proportions 51, 10, 35 and 4 respectively. The high recovery of carbon in the starch digested (96%) and the hydrogen/oxygen index for the fermentation products (2·12) suggest that the 'other gas' is mainly hydrogen.

Manometric experiments showed that glucose and maltose were not fermented by starved protozoa, nor were the sugars apparently converted into storage starch in the cells. Suspensions of protozoa were incubated for 10 hr. with 0·2% glucose or maltose; the concentration of sugar in the solutions remained unchanged, while the starch (measured as glucose) in the cells disappeared at the same rate as it did from suspensions incubated without added

sugar. The entodinia seemed more tolerant of acid conditions than the rumen holotrich protozoa, and remained active at pH 5·6.

REFERENCES

Coleman, G. S. (1958). *Nature, Lond.*, **182**, 1104.
Eadie, J. M. & Oxford, A. E. (1957). *Nature, Lond.*, **179**, 485.
Gutierrez, J. (1955). *Biochem. J.* **60**, 516.
Heald, P. J. & Oxford, A. E. (1953). *Biochem. J.* **53**, 506.
Howard, B. H. (1959*a*). *Biochem. J.* **71**, 671.
Howard, B. H. (1959*b*). *Biochem. J.* **71**, 675.
Oxford, A. E. (1958). *N.Z. J. Agric. Res.* **1**, 809.
Sugden, B. & Oxford, A. E. (1952). *J. gen. Microbiol.* **7**, 145.

Coenzyme Reduction by Illuminated Chloroplasts. By H. E. Davenport. (*A.R.C. Unit of Plant Nutrition (Micro-Nutrients), Long Ashton Research Station, University of Bristol*)

San Pietro & Lang (1958) have shown that illuminated chloroplasts will bring about a measurable reduction of coenzymes I and II (Co I and Co II) only in the presence of a soluble protein factor from leaves which they purified 35-fold and named 'photosynthetic pyridine nucleotide reductase'. Compared with rates of reduction of such artificial hydrogen acceptors as ferricyanide or 2:6-dichlorophenol indophenol in the chloroplast reaction, expressed on the basis of added chlorophyll, the maximum rates reported by San Pietro & Lang were low ($Q_{O_2}^{chl} = 190$). By modification of the photochemical system and further purification of the leaf protein, reduction rates comparable with those observed with the artificial hydrogen acceptors have now been obtained.

Minced pea leaves were fractionally precipitated with cold acetone as described by San Pietro & Lang. The redissolved active precipitate was then further fractionated with ammonium sulphate. Activity was found to be confined to the fraction precipitated between 80 and 100% saturation. It has already been shown that this pea leaf protein fraction is active in catalysing the reduction of methaemoglobin by illuminated chloroplasts (Davenport, Hill & Whatley, 1952). More recently the 'methaemoglobin reducing factor' has been obtained as a purified protein, homogeneous electrophoretically and in the ultracentrifuge and having a calculated molecular weight of 19 000 (Davenport & Hill, 1959). Preparations of this purified protein from the leaves of pea, spinach, parsley and beet have now been found to be highly active in the catalysis of Co II reduction ($Q_{O_2}^{chl} = 1000$). No activity towards Co I was detected. The reaction rate was found to be still further increased when coenzyme reduction was coupled to photophosphorylation by including a phosphate-acceptor system (adenosine diphosphate, inorganic phosphate, Mg) in the reaction mixture. With the complete reaction mixture and purified pea leaf protein the highest $Q_{O_2}^{chl}$ so far observed is 2700. The presence of the phosphate-acceptor system was without effect on the rate of reduction of methaemoglobin but Co II was inhibitory in this reaction.

REFERENCES

Davenport, H. E. & Hill, R. (1959). Submitted to *Biochem. J.*
Davenport, H. E., Hill, R. & Whatley, F. R. (1952). *Proc. Roy. Soc.* B, **139**, 346.
San Pietro, A. & Lang, H. M. (1958). *J. biol. Chem.* **231**, 211.

Lipoprotein of High Lipid Content from Egg Yolk. By W. M. McIndoe. (*A.R.C. Poultry Research Centre, Edinburgh* 9)

Most methods for fractionating yolk proteins (see Warner, 1954) involve the arbitrary use of ether for the extraction of what is alleged to be free lipid. Current workers (Sugano, 1958; Turner & Cook, 1958) are aware that ether may degrade native lipoproteins to give products of lower lipid content and altered physicochemical properties and Schmidt, Bessman, Hickey & Thannhauser (1956) and Evans & Bandemer (1957) conclude that no free lipid exists in yolk. In the present work the fractionation is performed without ether extraction and at low ionic strengths.

Yolk from fresh eggs is diluted with 0·15 M-NaCl and centrifuged free of particulate material, which contains about 5% of the lipid and 40% of the protein of yolk and virtually all the phosphoprotein in agreement with Schmidt *et al.* (1956). If the supernatant is diluted with water precipitation is just apparent at a dilution of 1 in 4 and is complete at about 0·02 M-NaCl. The precipitate (from a 1 in 10 dilution or by dialysis) is allowed to flocculate at 2° and can be centrifuged to the surface and reprecipitated from solution in 0·15 M-NaCl. The material contains 12% protein (only a trace of

phosphoprotein being present) and 88% lipid of which about 25% is phospholipid and 4% is cholesterol. It accounts for almost 95% of the yolk total lipid and cholesterol, about 90% of the phospholipid, and 45% of the protein.

A similar fraction has been obtained from laying hen plasma by the same procedure (McIndoe, 1959). However, the yolk fraction differs from this in some respects: it is not water soluble, contains less protein and practically no phosphoprotein. Also, in the analytical ultracentrifuge it rises rapidly at sp.gr. 1·006 tending to separate into two but showing no evidence of a sedimenting component. It is likely that the yolk fraction is related to the low density component of the complex obtained from plasma and further evidence on this will be presented.

Ether extracts 90% of the lipid, including all the cholesterol and 70% of the phospholipid, from a 0·15M-NaCl solution of the yolk lipoprotein. Of the unextracted material half is insoluble and almost all the rest is precipitated on dialysis. These fractions contain about 40% lipid at least 90% of which is phospholipid; the protein has 0·2% phosphorus, possibly representing firmly bound phospholipid (McIndoe, 1958).

REFERENCES

Evans, R. J. & Bandemer, S. L. (1957). *J. Agric. Fd Chem.* **5**, 868.

McIndoe, W. M. (1958). *Biochem. J.* **70**, 8P.

McIndoe, W. M. (1959). *Biochem. J.* **72**, 153.

Schmidt, G., Bessman, M. J., Hickey, M. D. & Thannhauser, S. J. (1956). *J. biol. Chem.* **223**, 1027.

Sugano, H. (1958). *J. Biochem.* (*Japan*), **45**, 667.

Turner, K. J. & Cook, W. H. (1958). *Canad. J. Biochem. Physiol.* **36**, 927.

Warner, R. C. (1954). *The Proteins*, p. 435, vol. II, pt. A. New York: Academic Press Inc.

The Effect of Sheep Rumen Contents on Triglycerides *in vitro*. By G. A. Garton, A. K. Lough and E. Vioque.* (*Rowett Research Institute, Bucksburn, Aberdeen*)

The presence of considerable amounts of stearic acid in the depot glycerides of ruminant animals has been attributed (e.g. Reiser & Reddy, 1956; Shorland, Weenink, Johns & McDonald, 1957) to the hydrogenating action of rumen bacteria on dietary C_{18} unsaturated fatty acids, especially linolenic acid, of which pasture glycerides are a rich source. In addition to hydrogenation, sheep rumen micro-organisms can also hydrolyze triglycerides *in vivo* and *in vitro* (Garton, Hobson & Lough, 1958).

In further *in vitro* experiments sheep rumen contents were incubated anaerobically at 37° for 24 hr. with and without the addition of linseed oil (iodine value 181·1; 1·0 g./100 ml. rumen contents). After incubation about 95% of the esterified fatty-acid residues of the oil were accounted for as free higher fatty acids, the composition of which was determined by gas chromatography using polymerized ethylene glycol adipate as liquid phase and argon as carrier gas. The respective compositions of the linseed oil and of the free fatty acids produced during the incubation were (in mols. %): palmitic 5·6 and 13·8, stearic 5·8 and 31·7, oleic 21·6 and 30·2, linoleic 12·5 and 14·2, linolenic 54·5 and 5·1, unidentified acids (two) 0·0 and 5·0. It can be concluded from the work of Shorland *et al.* (1957) that unsaturated acids found after incubation include spatial and positional isomers of those present in the original oil.

The unsaturated acids, especially linolenic acid, had thus undergone extensive hydrogenation giving rise to increased amounts of stearic acid; the unexpectedly increased amount of palmitic acid possibly resulted from the breakdown of stearic acid. The two unidentified acids (having a chain length less than C_{16}) were also present in the free, non-volatile fatty acids of rumen contents *per se*, but increased amounts were found following the incubation of linseed oil.

Under similar conditions olive oil (iodine value 80·4) was hydrolyzed to the extent of about 70% of its component acids. The free fatty acids formed contained none of the linoleic acid present initially in the oil; stearic acid was again present in quantity and palmitic acid was produced together with small amounts of the same two unidentified fatty acids which resulted from the incubation of linseed oil.

Cocoa butter (iodine value 39·9) incubated under the same conditions with rumen contents resulted in the liberation of about 40% of its component acids. It seems that the extent to which triglycerides are hydrolysed in rumen contents may be related to the degree of unsaturation of their component acids.

* Juan March Foundation Fellow on leave from the Instituto de la Grasa y sus Derivados, Seville, Spain.

REFERENCES

Garton, G. A., Hobson, P. N. & Lough, A. K. (1958). *Nature, Lond.*, **182**, 1511.

Reiser, R. & Reddy, H. G. R. (1956). *J. Amer. Oil Chem. Soc.* **33**, 155.

Shorland, F. B., Weenink, R. O., Johns, A. T. & McDonald, I. R. C. (1957). *Biochem. J.* **67**, 328.

The Nutritional Requirements of *Actinomyces israeli*. By A. O. CHRISTIE and J. W. PORTEOUS. (*Department of Biological Chemistry, Marischal College, University of Aberdeen*)

The anaerobe, *Actinomyces israeli*, is nutritionally a reputedly fastidious organism. Erikson & Porteous (1953) attempted the first simplification of the classical complex undefined media generally used for culturing the organism and obtained a maximum cell yield (equivalent to 17 μg. total nitrogen) from each millilitre of a broth/casein hydrolysate medium containing approximately 2000 μg. total nitrogen. These authors failed to replace the broth successfully by synthetic nutrients but concluded that the organism could be cultured for prolonged periods in relatively simple media without the diminished vitality frequently reported when complex media are used (Erikson, 1940, 1949).

Howell & Pine (1956) found that five strains of *A. israeli* would grow in a medium elaborated initially to support *Histoplasma capsulatum*; this medium contained inorganic salts, twenty amino acids, glutathione, five purine and pyrimidine bases, seventeen growth factors, starch, oleic acid and glucose.

In the present work twelve strains of *A. israeli*, all isolated from human actinomycotic lesions, have been studied, one of them ('Wills') in detail. In the original Howell and Pine medium a cell yield of 50 μg. total nitrogen was obtained with this strain from each millilitre of medium containing 1070 μg. total nitrogen. The medium has been simplified so that it contains inorganic salts, twenty amino acids, five growth factors (nicotinic acid, riboflavine, pyridoxal, biotin and inositol) and glucose; the total nitrogen content is now 300 μg./ml. and the cell yield in this completely synthetic medium has been concomitantly increased to approximately 80 μg. total nitrogen/ml. The minimal amino acid requirements appear to be glutamic acid, serine, cysteine, leucine, *iso*leucine, lysine and tryptophan.

Of the remaining eleven strains, five survive in continued culture in a slight modification of the Howell and Pine medium. Two of these (in contradistinction to the 'Wills' strain), appear to require starch. The results suggest strain differences in the nutritional requirements of *A. israeli*.

A search for amino acid decarboxylases and deaminases in the organism has led to negative results; the Stickland reaction is absent; growing cells do not hydrolyse external supplies of starch and do not exhibit extracellular proteolytic activity. The formation of lactic acid as the major end-product of carbohydrate metabolism (Erikson & Porteous, 1953) has been confirmed.

REFERENCES

Erikson, D. (1940). *Spec. Rep. Ser. med. Res. Coun., Lond.*, no. 240.
Erikson, D. (1949). *Ann. Rev. Microbiol.* **3**, 23.
Erikson, D. & Porteous, J. W. (1953). *J. gen. Microbiol.* **8**, 464.
Howell, A. & Pine, L. (1956). *J. Bact.* **71**, 47.

The Kinetic Properties of Invertases Obtained from Different Genera of Sheep-rumen Holotrich Protozoa. By J. A. CARNIE and J. W. PORTEOUS. (*Department of Biological Chemistry, Marischal College, University of Aberdeen*)

Christie & Porteous (1957) reported on a crude invertase from protozoal isolates containing the three species *Dasytricha ruminantium*, *Isotricha intestinalis* and *I. prostoma* in unknown and possibly varying proportions. The results then obtained may be summarized as follows: apparent hydrolytic activity of the crude enzyme rose sharply with increasing substrate concentration reaching a sharp maximum at 0·025M-sucrose, then decreased gradually until at 0·1M-sucrose it approached 70% of the peak value and remained at this level for all higher sucrose concentrations up to 1·0M; oligosaccharide formation was not apparent below 0·1M-sucrose and appeared to reach a maximum at 0·25M-sucrose.

It has now been possible to examine the two genera separately although the *Isotricha* isolates still consisted of two species in unknown proportions; the techniques used were those previously reported (Christie & Porteous, 1957).

The apparent hydrolytic activity/substrate concentration curve for the extract from the two *Isotricha* species was essentially identical with that obtained for the three mixed species (Christie & Porteous, 1957); chromatographic analysis also gave results essentially identical with those already reported.

The apparent hydrolytic activity/substrate concentration curve for the *Dasytricha* extract, in complete contrast to the *Isotricha* and the mixed-genera curves, rose smoothly from zero activity to approach a maximum at 0·1M-sucrose with no peak or discontinuity in the curve; oligosaccharide formation occurred at the same approximate

degree of hydrolysis of the same sucrose concentrations as with the *Isotricha* and mixed-genera extracts.

The conclusion drawn from the previous work that the protozoal invertase shows markedly less transferring activity than any other invertase is substantiated but the present results suggest the presence of species-specific invertases in the original extracts and it seems very probable that the substrate concentration/hydrolytic activity curve previously reported was a resultant curve derived from three separate invertases showing similar transferring activities but different kinetic properties in respect of their hydrolytic activities. Howard (1959) has reported on other differences between the protozoal enzymes and has identified the products of the action of the *Isotricha* enzyme on sucrose.

We are indebted to Dr B. H. Howard for a gift of protozoal isolates.

REFERENCES

Christie, A. O. & Porteous, J. W. (1957). *Biochem. J.* **67**, 19P.
Howard, B. H. (1959). *Biochem. J.* **71**, 675.

Rabbit Intestinal Invertase. By J. A. Carnie and J. W. Porteous. (*Department of Biological Chemistry, Marischal College, University of Aberdeen*)

Centrifugal fractionation in 0·25M-sucrose of disintegrated mucosal cells of rabbit intestine yielded a microsome preparation (Fraction 1) with invertase activity. Treatment of an aqueous suspension of microsomes with *n*-butanol led to complete loss of activity. Treatment with deoxycholate followed by dialysis gave Fraction 2 with a 1·7-fold increase in invertase activity; 78% of this activity was in the supernatant (Fraction 3) after centrifuging Fraction 2 at 105 000 *g* for 60 min., the remainder being recovered in the sediment. Treatment with deoxycholate followed by treatment with *n*-butanol and removal of the butanol and the interface precipitate gave, after dialysis, Fraction 4 containing 59% of the activity of Fraction 2. 60% of the activity of Fraction 4 remained in the supernatant (Fraction 5) after centrifuging as before, the remaining activity being recovered in the pellet. The specific activity of Fraction 5 was 2·5 times that of Fraction 3.

Fractions 3 and 5 appear to represent the microsomal invertase in true solution. The former fraction tended to flocculate on incubation at 37° under optimal assay conditions; the latter remained water clear.

Hydrolase and transferase activity of Fractions 3 and 5 were determined as described by Christie & Porteous (1957). With sucrose as substrate in 0·04M-phosphate buffer at 37° the optimal pH was 6·7, the optimal substrate concentration 0·1M, $K_m = 6{\cdot}2 \times 10^{-3}$.

Fractions 3 and 5 produced a single 'upper spot' on paper chromatograms (Bealing & Bacon, 1953) after incubation under optimal hydrolase assay conditions. The amount of 'upper spot' material formed increased with increasing sucrose concentration and accounted for 4% of all sugars present at 20% hydrolysis of 0·5M-sucrose. The oligosaccharide ($R_s = 0{\cdot}6$) showed no reducing properties and contained equimolar amounts of glucose and fructose. Ethanol (10%, v/v) as additional substrate to 0·1 and 0·5M-sucrose failed to produce ethylglucoside or ethylfructoside (cf. Bacon, 1952; Bealing, 1953; Christie & Porteous, 1957). Maltose was hydrolysed at approximately the same rate as sucrose and two 'upper spots' were produced from maltose neither of which was identical with the material obtained from sucrose.

The apparently restricted transferase activity of the intestinal invertase may be due to the maltase activity of the partly purified enzyme.

REFERENCES

Bacon, J. S. D. (1952). *Biochem. J.* **50**, xviii.
Bealing, F. J. (1953). *Biochem. J.* **55**, 93.
Bealing, F. J. & Bacon, J. S. D. (1953). *Biochem. J.* **53**, 277.
Christie, A. O. & Porteous, J. W. (1957). *Biochem. J.* **67**, 19P.

THE

JOURNAL OF PHYSIOLOGY

NOVEMBER 1959. VOL. 148, NO. 3

CAMBRIDGE, G. W., HOLGATE, J. A. and SHARP, J. A. A pharmacological analysis of the contractile mechanism of *Mytilus* muscle.

DABASH, A. and SHARAF, A. The response of canine endometrium to various hormones and related compounds.

PIERCE, A. E. Studies on the proteinuria of the new born calf.

THOMAS, S. Effects of change of posture on the diurnal renal excretory rhythm.

KAY, R. N. B. and PHILLIPSON, A. T. Responses of the salivary glands to distension of the oesophagus and rumen.

HARKNESS, MARGARET L. R. and HARKNESS, R. D. Changes in the physical properties of the uterine cervix of the rat during pregnancy.

DE LA LANDE, I. S. and WHELAN, R. F. The effect of antagonists on the response of the forearm vessels to adrenaline.

CROSS, B. A. and GREEN, J. D. Activity of single neurones in the hypothalamus: effect of osmotic and other stimuli.

BLASCHKO, H. Oxidation of 1,4-methylhistamine by mammalian plasma.

HUBEL, D. H. and WIESEL, T. N. Receptive fields of single neurones in the cat's striate cortex.

COLLINS, K. J., SARGENT, F. and WEINER, J. S. Excitation and depression of eccrine sweat glands by acetylcholine, acetyl-β-methylcholine and adrenaline.

COLLINS, K. J., SARGENT, F. and WEINER, J. S. The effect of arterial occlusion on sweat-gland responses in the human forearm.

LLOYD, SYBIL. The vascular responses of the rat during the reproductive cycle.

BLAIR, D. A., GLOVER, W. E., GREENFIELD, A. D. M. and RODDIE, I. C. Excitation of cholinergic vasodilator nerves to human skeletal muscles during emotional stress.

BLAIR, D. A., GLOVER, W. E. and RODDIE, I. C. The abolition of reactive and post-exercise hyperaemia in the forearm by temporary restriction of arterial inflow.

THESLEFF, S. Motor end-plate 'desensitization' by repetitive nerve stimuli.

DEL CASTILLO, J. and MOORE, J. W. On increasing the velocity of a nerve impulse.

FRANKENHAEUSER, B. Steady state inactivation of sodium permeability in myelinated nerve fibres of *Xenopus laevis*.

FRANKENHAEUSER, B. and WALTMAN, B. Membrane resistance and conduction velocity of large myelinated nerve fibres from *Xenopus laevis*.

BROWN-GRANT, K. and PETHES, G. Concentration of radio-iodide in the skin of the rat.

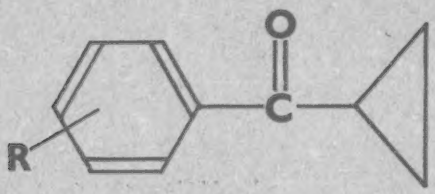
Subscription price 80s. per volume of three parts, post free

Single parts 30s. each, postage extra

CAMBRIDGE UNIVERSITY PRESS

BENTLEY HOUSE, 200 EUSTON ROAD,
LONDON, N.W.1

CYCLOPROPYL KETONES



4-BROMOPHENYL CYCLOPROPYL KETONE
4-tert.-BUTYLPHENYL CYCLOPROPYL KETONE
4-CHLOROPHENYL CYCLOPROPYL KETONE
CYCLOPROPYL 2,4-DIMETHYLPHENYL KETONE
CYCLOPROPYL 2,5-DIMETHYLPHENYL KETONE
CYCLOPROPYL 3,4-DIMETHYLPHENYL KETONE
CYCLOPROPYL 4-ETHYLPHENYL KETONE
CYCLOPROPYL 4-FLUOROPHENYL KETONE
CYCLOPROPYL 4-ISOPROPYLPHENYL KETONE
CYCLOPROPYL 4-METHOXYPHENYL KETONE
CYCLOPROPYL 4-METHYLPHENYL KETONE
CYCLOPROPYL PHENYL KETONE

We are the United States sales agent for the Research Laboratorium, Dr C. Janssen, the manufacturer of these compounds

References to the reactions of these ketones:

1. C. Allen and R. Boyer: *Can. J. Research*, **9**, 159 (1933).
2. S. Bunce and J. Cloke: *J. Am. Chem. Soc.*, **76**, 2244 (1954).
3. W. Close: *J. Am. Chem. Soc.*, **79**, 1455 (1957).
4. R. Fuson and F. Baumgartner: *J. Am. Chem. Soc.*, **70**, 3255 (1948).
5. K. Gitschthaler and W. Klementschitz: *Scientia Pharm.*, **23**, 79 (1955).
6. A. Haller and F. Benoist: *Ann. Chim.*, **17**, 25 (1921).
7. H. Henze and C. Gayler: *J. Am. Chem. Soc.*, **74**, 3615 (1952).
8. H. House and J. Blaker: *J. Org. Chem.*, **23**, 334 (1958).
9. N. Kizhner: *J. Russ. Phys. Chem. Soc.*, **43**, 1163 (1911).
10. F. La Forge: *J. Am. Chem. Soc.*, **50**, 2475 (1928).
11. P. Lipp, J. Buchkremer and H. Seeles: *Ann.*, **499**, 1 (1932).
12. W. Perkin: *J. Chem. Soc.*, **47**, 840 (1885).
13. T. Marshall and W. Perkin: *J. Chem. Soc.*, **59**, 853 (1891).
14. F. Piehl and W. Brown: *J. Am. Chem. Soc.*, **75**, 5023 (1953).

These twelve aromatic cyclopropyl ketones, as well as cyclopropyl 2-thienyl ketone, cyclopropyl methyl ketone and dicyclopropyl ketone are available from stock.

Write for Catalog No. 9

ALDRICH CHEMICALS

Listing over 4000 research chemicals

ALDRICH CHEMICAL COMPANY, INC.

2369 NORTH 29TH STREET · MILWAUKEE 10, WISCONSIN, U.S.A.

British Sales Agent: RALPH N. EMANUEL Ltd., 3 Leather Market, Weston St., London, S.E.1
Tel. Hop. 3937

Printed in Great Britain at the University Press, Cambridge
(Brooke Crutchley, University Printer)